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Invasive silver carp (Hypophthalmichthys moltrix) protein hydrolysates and their efficacy in increasing seedling vigor and reducing seed borne pathogen growth

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INVASIVE SILVER CARP (*Hypophthalmichthys moltrix)* PROTEIN HYDROLYSATES AND THEIR EFFICACY IN INCREASING SEED VIGOR AND REDUCING SEED BORNE PATHOGEN GROWTH

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Dedicated to the search for coffee creamer, fortuitous office placements and supportive parents everywhere

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ABSTRACT

Thomson, Shanleigh P. M.S. Purdue University, December 2014. Invasive Silver Carp (*Hypophthalmichthys* moltrix) protein hydrolysates and their efficacy in increasing seed vigor and reducing plant pathogen growth. Major Professor: Andrea Liceaga.

Silver carp are an invasive fish species introduced into the United States within the last forty years. Unable to be effectively eradicated or commercialized as a source of food, other methods must be developed to control and/or use these fish. Fish protein hydrolysates (FPH) are products derived from the proteins inherent within fish. Development of FPH from Silver carp for a variety of uses could aid in controlling their expanding population and provide organic produces with a seed treatment option which is not available to them currently.

In the first study, FPH were used as a seed vigor treatment for soybeans and watermelon seeds. Seedlings were treated with FPH hydrolyzed for 1, 5.5 and 10 hours with papain (FPH-Pa), pepsin (FPH-P) and trypsin (FPH-T), respectively. Overall vigour tests (accelerated aging and warm and cold germination dry weight, height, total phenolics and guaiacol peroxidase assessment) were compared to a distilled-water control over a 12-day germination period. Soybean seeds treated with FPH-P and FPH-Pa at 1 hour elicited the greatest growth responses with increased total percent germination, and higher seedling weights and lengths. GuPx values were also higher in FPH treated soybeans. Results suggest that FPH is beneficial to the stimulation of the proline-linked pentose phosphate

pathway (PPP) in soybeans, enhancing vigor parameters measured. In watermelon seedlings, FPH treatments increased all parameters with the exception of total phenolic content as compared to water controls, indicating the PPP was not stimulated. The amino acids TRP, MET, PRO, TYR, THR, VAL, LYS and ASP appear to stimulate growth.

CHAPTER 1. LITERATURE REVIEW

1.1 Asian Carp in the US: History Influence, Control and Uses

Introducing foreign species, including fish, into local ecosystems has been employed for a variety of purposes with different degrees of success (Pimentel, Zuniga, & Morrison, 2005). Beginning in the 17th century, fish have been consistently imported into US waterways from around the world to carry out specific functions (Gido & Brown, 1999), including a desire by immigrants for food from their home countries, the replenishment of fish quantities and enhancement of water quality in ecosystems degraded by human irresponsibility and an increasing game-fish industry (Crossman, 1991). An increase in established foreign fish species occurred in the 20th century, and by the late 1980's, the number had risen from five species in 1955 to 45 (Crossman, 1991). Currently, there are 138 established foreign fish species in the US that are deemed invasive, accounting for $5.4 billion per year in economic losses (Pimentel, Zuniga, & Morrison, 2005). Of these, Asian carp species have been some of the most detrimental causing damage to both environmental ecosystems and humans (Minnesota DNR, 2005).

Asian carp include the species *Hypophthalmichthys noblis* (Bighead Carp) and *Hypophthalmichthys moltrix* (Silver Carp) (Schofield, Williams, Nico, Fuller, & Thomas, 2005). In the early 1970s, both carp species were introduced into aquacultural production ponds to remove algae and plankton, in addition to cleaning the water (Freeze &

Henderson, 1982). Further evaluation of the efficacy of these species for expanded use in larger aquacultural facilities lead to their escape into US waterways (Schofield, Williams, Nico, Fuller, & Thomas, 2005). Asian carp now have been reported in 18 states; both species are established in Illinois and Missouri and have expanded exponentially in Indiana (Schofield, Williams, Nico, Fuller, & Thomas, 2005).

Asian carp species are now considered "Injurious wildlife" (Osteen, Gottlieb, & Vasavada, 2012) by the US Fish and Wildlife Service for a number of reasons.
First,, Asian carp species are highly fecund and can produce up to 2 million eggs per fish (Schofield, Williams, Nico, Fuller, & Thomas, 2005), allowing them to reproduce quickly and in large quantities creating a competitive advantage over native species. Secondaly, Asian carp grow to 1 meter in length and can weigh up to 100 lbs (39 kg) and, because of their sheer size, they do not allow native species to easily co-exist in a typical river system (Nico & Fuller, 2012). The high population density of the Asian carp also causes many safety issues for motorists and pleasure craft operators. Kolar et al , 2005 report that the fish respond to an outboard motor by jumping out of the water and hitting people causing injury. Carp are planktivores and feed at low trophic levels (Ruebush, 2011). Studies have indicated that certain native fish species compete with Asian carp for food because of their similar diets (Irons, Sass, McClelland, & Stafford, 2007; Williamson & Garvey, 2005). The concern many ecologists have is the potential of the elimination of native fish species by the direct competition for food (Kolar, Chapman, Courtenay Jr, Housel, Williams, & Jennings, 2005). The aggressive nature of the Asian carp has also raised concerns over the loss of biodiversity in the Mississippi River and other ecosystems (Kolar, Chapman, Courtenay Jr, Housel, Williams, & Jennings, 2005).

The control of these fish has thus far been unsuccessful and there is no current method to eradicate them (Kolar, Chapman, Courtenay Jr, Housel, Williams, & Jennings, 2005). Previous studies conducted in China on both species of carp, same to those found in the Wabash River in Indiana, indicated that they are able to withstand high levels of chemical exposure due to their adaptation to the toxic conditions of the Yangtze River (Ryans & Environmental Research, 1990); indicating their heartiness and ability to thrive under adverse conditions. Alternative chemical and electro-chemical methods are currently being explored as means of controlling the population, yet no permanent methods have been developed or employed (Minnesota DNR, 2005).

Locally, Asian carp populations are rapidly expanding in the Wabash River, and currently comprise sixty-three percent of the total fish biomass in the Illinois River (Roth & Secchi, 2012). A study conducted by the Asian Carp Regional Coordinating Committee (ACRCC) indicates Asian carp contribute anywhere between 3 and 10 million pounds of biomass (2012). Recommendations by the ACRCC for control of Asian carp population primarily relate to the harvest and use of these fish rather than picicidal options that many other organizations suggest. Carp have very high protein content (Roth & Secchi, 2012), and according to the Food and Agriculture Organization of the United Nations, (FAO, 2012), Silver Carp are priced at approximately $0.50/kg in China. This is a much lower market value compared to other fish commonly consumed and harvested in the United States (FAO, 2012). Despite a reasonable purchase price and excellent nutritional profile, exploration into manufacturing the fish into secondary products or using them as fish fillets has not been well received (Roth & Secchi, 2012). Reasons for the poor consumer acceptance of this fish are due to the fish are too bony to efficiently

produce secondary products; consumers of the fillets indicated dissatisfaction associated with the high bone content (Freeman, 1999; Roth & Secchi, 2012). Thus alternative uses for the fish must be examined.

1.2 Fish Protein Hydrolysates (FPH)

Hydrolyzing muscle protein derived from underutilized fish and fish waste is not only an excellent use of these resources but also provides a wide range of potentially functional products (Kristinsson & Rasco, 2000). Protein hydrolysates are defined as the products of a hydrolysis reaction (i.e. peptides or peptones, amino acids and residual lipids, carbohydrates and minerals from the reaction) through the use of enzymes, acids or alkalis (Pasupuleti & Braun, 2010). The chemical, physicochemical and functional properties of the products produced are dependent on the protein source and the method used to obtain the protein hydrolysates, thus, giving FPH a variety of potential uses (Pasupuleti & Braun, 2010). Initially, meat hydrolysates were used solely as a growth medium for microbes. Over time, the uses for hydrolysates from other protein sources expanded to include functional ingredients for animal and human nutritional benefits (e.g. emulsifiers, stabilizers, antioxidants, starter culture, etc) (Pasupuleti & Demain, 2010). Aside from uses in food systems, protein hydrolysates are currently being examined for possible use in an agronomic setting as a natural herbicide and fertilizer (Cavani, Ter Halle, Richard, & Ciavatta, 2006; J. Yang & Lu, 2010).

Initially, hydrolysates were not easily produced due to the length of time, cost and complicated nature of the procedures used to procure protein hydrolysates (Adler-Nissen, 1976). New methods for creating protein hydrolysates have been developed. Advances

primarily associated with novel enzyme production and fermentation processes have allowed for the creation of higher quality protein hydrolysates, specific for new applications, to be created in a more efficient and cost effective manner (Pasupuleti & Braun, 2010). With this in mind, the production of fish protein hydrolysates (FPH) from the fish waste in industry, in addition to the protein found in Asian carp, is an acceptable means of creating new, functional products from an under-utilized source.

1.3 Previous Investigations of Fish Protein Hydrolysates

1.3.1 Functional Properties

Preliminary examinations into the functionality of fish proteins were conducted in the 1960s on fish protein concentrates (FPC); protein components extracted through solvent methods (Hoyle & Merritt, 1994). FPCs were low in functionality (i.e. they were not soluble) but highly nutritive, which led to the development of FPH as a more functional ingredient for use as a cheap protein additive in animal feeds and the food industry (Hoyle & Merritt, 1994; Kristinsson & Rasco, 2000). Early issues relating to bitterness of the FPH and high economic costs prevented wide spread use of FPH (Kristinsson & Rasco, 2000). Advanced techniques, such as varying the degree of hydrolysis (time in which the protein is exposed to the acid, alkali or enzyme) and using different enzymes and pH ranges have expanded the functionality of FPH for use in food systems, increasing solubility, emulsification capacity and foaming ability (Liceaga-Gesualdo & Li-Chan, 1999; Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suárez, 2008). FPH are highly soluble due to the nature of their hydrophilicity and ionic interactions with solutions (Kristinsson & Rasco, 2000). In one study, all FPH of Pacific

Whiting produced at various degrees of hydrolysis were found to have solubility of over 97% that was attributed to the low molecular weight of the FPH (Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suárez, 2008). Solubility of FPH also affects the other functional abilities of FPH. Studies relating to the emulsifying properties, including emulsification activity (as represented by the emulsifying activity index [EAI]) and emulsification capacity (EC) have demonstrated that FPH are excellent emulsifiers compared to the un-hydrolyzed fish proteins (Cheung, Liceaga, & Li-Chan, 2009; Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suárez, 2008). Results from these studies also have concluded that FPH have good foamability (ability to form a foam when air is incorporated into a hydrolyzed protein solution). As the molecular weight of the peptides in the FPH deceases, however, the stability of the foam decreases. As the molecular weight of the peptides in FPH decreases, they may require additional stabilizers to maintain a foam.

1.3.2 Bioactive Components

Investigations into bioactive properties of FPH are a more recent development in the scientific community, as the benefits of such components (e.g. antioxidant properties, ACE-inhibitory,) are now more thoroughly understood (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; Raghavan & Kristinsson, 2009). Antioxidant peptide fractions have been isolated from many different fish. Hydrolyzed silver carp and grass carp muscle proteins contain low molecular weight peptide fractions (<1 kDa) that exhibit very high antioxidant capabilities (Ren, Zhao, Shi, Wang, Jiang, Cui, et al., 2008; Zhong, Ma, Lin, & Luo, 2011). Studies regarding angiotensin converting enzyme (ACE)

inhibitory effects of FPH from fish such as *Oreochromis niloticus* (Tilapia) and *Sardinella longiceps* (Sardinelle) demonstrate lower MW peptide fractions (<10 kDa) exhibit high ACE-inhibitory effects (Bougatef, Nedjar-Arroume, Ravallec-Plé, Leroy, Guillochon, Barkia, et al., 2008; Raghavan & Kristinsson, 2009). Peptide fractions that exhibit antioxidant properties in silver carp had a high content of hydrophobic amino acids that allowed for increased solubility in hydrophobic targets such as lipids (Zhong, Ma, Lin, & Luo, 2011). This property of antioxidant peptides has been confirmed by other studies conducted with several other fish species, including *Limanda aspera* (yellowfin sole), *Johnius belengerii* (hoki) and *Theragra chalcogramma* (Alaska Pollack) (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012). ACE-inhibitory peptides typically contain hydrophobic amino acids, in addition to a hydroxyproline residue required for binding to ACE, and a positively charged amino acid at the C-terminus (Jung, Mendis, Je, Park, Son, Kim, et al., 2006; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). The active sites of ACE have been found to be largely hydrophobic in nature and therefore hydrophilic peptides are not as effective as inhibitors of this enzyme (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011).

Isolation and purification of such specific peptide fractions are more easily done through newer enzymatic technologies that allow for processors to utilize these inherent properties (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012). In a number of different countries the marketing of nutraceuticals and functional foods containing FPH that enhance gastrointestinal health, cognitive function, metabolism and blood pressure is common, however, there have been limited clinical studies as to how these

products affect human health (Boutin, Paradis, Couture, & Lamarche, 2012; Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012).

1.3.3 Cryoprotectant Properties

Proteins and FPH have been examined for their cryoprotectant properties, that is, their ability to prevent deterioration of proteins during frozen storage (Cheung, Liceaga, & Li-Chan, 2009; Dey & Dora, 2011; Yoon, Lee, & Hufnagel, 1991). Cheung, Liceaga and Li-Chan (2009) reported that FPH with higher Asp, Glu, Arg and Lys content were better cryoprotectants. FPH with a highly hydrophobic surface also is attributed to have a greater cryoprotectant affect (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2012). Size of the peptides did contribute to some of the cryoprotectant properties; oligopeptides were better cryoprotectants than polypeptides but this was not as important as the amino acid composition of the peptide fractions (Cheung, Liceaga, & Li-Chan, 2009). Proteins, and protein fractions have been shown to be good alternatives to the commercially available chemical cryoprotectants in the preservation of prepared and frozen food products such as surimi (minced fish) (Cheung, Liceaga, & Li-Chan, 2009; Dey & Dora, 2011; Ishiguro, 1998; Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2012; Yoon & Lee, 1990; Yoon, Lee, & Hufnagel, 1991).

1.3.4 Antimicrobial Properties of Proteins and Peptides

Antimicrobial protein fractions have been identified in a number of different plant and animal sources. The most extensively studied proteins with bactericidal properties are peptides derived from milk and eggs, such as lactoferrins from milk and lysozyme

and albumin hydrolysates from eggs (Di Bernardini, Harnedy, Bolton, Kerry, O'Neill, Mullen, et al., 2011).

1.3.5 Milk Antimicrobial Peptides

Lactoferrin is typically digested using pepsin and, in some circumstances, chymosin, creating the antimicrobial peptides lactoferricin H and B. The mechanisms by which they operate and their molecular properties have been detailed in numerous studies (Bellamy, Takase, Wakabayashi, Kawase, & Tomita, 1992; Wakabayashi, Yamauchi, & Takase, 2006). Lactoferrin peptides that exhibit antimicrobial growth are cationic in nature and contain tryptophan and basic amino acid residues. They are most potent in a looped conformation of 11 residues (Farnaud & Evans, 2003). In Lactoferrin H (the peptide derived from human milk), spatial confirmation of the peptide (i.e. maintaining the looped region of the peptide fraction through proline residues) is more important than the composition (Farnaud & Evans, 2003; Hancock & Chapple, 1999). Lactoferrins inhibit microbial growth by binding to the lipopolysacharrides on bacterial cell membranes of gram negative bacteria, leading to the disruption within the cytoplasm of essential anionic molecules such as RNA and DNA, and preventing the uptake of iron essential for metabolism (Farnaud & Evans, 2003). Sequestering of ferric ions can only be completed by native lactoferrin molecules through a non-siderophone mediated mechanism (Gonzalez, Caamano, & Schryvers, 1990).

α -Lactalbumin is an acidic peptide present in whey protein. It is hydrolyzed by trypsin and chymotrypsin to three small functional bacteriocidal peptides (A. Pellegrini,

2003). The unique aspect of **α**-Lactalbumin peptides as compared to other antibacterial proteins is their ability to act against gram-positive microorganisms (A. Pellegrini, 2003). It appears the mechanism by which they operate is through is their hydrophobic residues, binding metal cations and interacting with lipid membranes within the gram-positive organisms (Permyakov & Berliner, 2000).

1.3.6 Egg Antimicrobial Peptides

Lysozyme is derived from egg white, but can also be found in human tears (A. Pellegrini, 2003). Native lysozyme exhibits catalytic activity, hydrolyzing the peptidoglycan cell wall layer found in prokaryotic organisms but specifically in gram-positive bacteria. Lysozyme can be hydrolyzed without losing antimicrobial abilities by numerous peptidases, most notably clostripain (A Pellegrini, Thomas, Bramaz, Klauser, Hunziker, & Von Fellenberg, 1997). Clostripain functions in a similar way to trypsin in that it cleaves after arginine residues, however, unlike trypsin, it does not cleave the peptide bond after lysine residues (A Pellegrini, Thomas, Bramaz, Klauser, Hunziker, & Von Fellenberg, 1997).

Other peptides extracted from egg white include ovotransferrin, an acidic glycoprotein approximately 68 kDa in size (A. Pellegrini, 2003). Ovo-transferrin operates in a similar way to lactoferrin, binding iron in addition to other metal ions such as zinc (Ibrahim, Iwamori, Sugimoto, & Aoki, 1998). Digestion with chymotrypsin yielded a large peptide containing 92 residues as a derivative of ovo-transferrin, which has been

found to damage the cytoplasmic membrane of the bacterium, *Escherichia coli* (Ibrahim, Iwamori, Sugimoto, & Aoki, 1998; Naidu, 2000)

1.4 Fish Antimicrobial Peptides

A number of peptides from fish have been examined and determined to be antimicrobial in nature (antimicrobial peptides or AMPs). Studies examined by Bao (2006) investigated antimicrobial properties of FPH obtained from the skin, intestinal, gill, liver, mucus and blood. Only (Park, Park, Kim, & Kim, 1998) examined loach as a whole fish for sources of antimicrobial peptides. AMPs are part of a highly specific system of defense that is elicited by the cells in different tissues (eg. Skin, gill, liver etc.) in response to foreign organisms such as bacterial cells (Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). Ravichandran et al. (2010) characterized peptides from different fish species .Extraction methods varied depending on what part of the fish was being extracted. Epithelial tissues, including cells from gills, skinand organ linings were carefully extracted using a scalpel and often acidified to prevent against degradation by proteolytic enzymes on the surface(Cole, Weis, & Diamond, 1997; Park, Park, Kim, & Kim, 1998; Shai & Oren, 2001). Mucosal analysis for AMPs involves electrocution of live fish and immediate collection of the layer of the external mucous layer (Gobinath & Ravichandran, 2011; Patrzykat & Douglas, 2003).

1.4.1 Characterization of Fish AMPs and Common Antimicrobial Mechanisms

There are many similarities amongst AMPs regardless of anatomical location or fish species. AMPs are small, averaging approximately 25 amino acid residues and

between 2000-3000 Da in size. The amino acid residues are predominantly basic in nature. The overall structure of the peptides displays amphipathic properties, important in the function against microorganisms through the interactions with membranes and membrane components (Powers & Hancock, 2003). The hydrophobicity of the peptide allows it to interact with lipids in the cell membranes. AMPs found in fish are most commonly a secondary alpha-helical structure. In this configuration, the peptide is able to bore holes into the bacterial membrane (Ravichandran et al. 2010; Bao et al., 2006; Bernardini et al., 2010). Beta-sheets and extended structures with trypsin, proline or histidine at the C-terminal also have been found in fish AMP conformations (Bao, Peatman, Xu, Li, Zeng, He, et al., 2006; Cole, Weis, & Diamond, 1997; Di Bernardini, et al., 2011; Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). Extended structures interact through hydrophobic and Van der Waals contact forces binding with the lipids present on pathogen membranes. The mechanism by which beta-sheets inhibit bacteria and other microorganisms has yet to be identified (Jenssen, Hamill, & Hancock, 2006; Powers & Hancock, 2003). AMPs are primarily cationic, which allows them to perform numerous functions when operating against invading pathogens (Jenssen, Hamill, & Hancock, 2006; Powers & Hancock, 2003). The cationic peptide binds to the anionic lipopolysaccharide in gram-negative bacteria or the techoic acids in gram-positive bacteria, disrupting the membrane and allowing the peptide to enter the monolayer between the two membranes. Further dissociation of the cytoplasmic membrane allows the peptide to enter the cytoplasm and, it is at this point, most peptides with alpha helical structures promote the leakage of essential cytoplasmic nutrients out of the bacteria cell (Jenssen, Hamill, & Hancock, 2006; Powers & Hancock, 2003). Other peptides with

specific amino groups, such as proline or arginine can interact with nuclear structures disrupting or altering DNA and RNA (Di Bernardini, et al., 2011; Jenssen, Hamill, & Hancock, 2006; Powers & Hancock, 2003). These peptides are able to interact via similar mechanisms with both viral envelopes and fungal cells (Jenssen, Hamill, & Hancock, 2006). Other mechanisms associated with AMP structure and chemical properties aid in the destruction of microorganisms through synergizing with autolytic enzyme systems, often inducing an autoimmune response in the invader (Hancock & Chapple, 1999; A. Pellegrini, 2003; Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010).

Ravichandran et al. (2010), summarized the anatomic locations of the most commonly found AMPs in marine fishes, as well as their average size and length. The primary location investigated for AMPs in fish are the external components, such as skin and mucous, as these areas are more likely to be in contact with foreign entities such as bacteria and fungi. Meat and muscle tissue of marine life has not been as extensively researched (Patrzykat & Douglas, 2003; Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010).

Further discussion of extraction and hydrolysis methods used to derive the peptide fractions from fish meat are included in the materials and methods section of this proposal.

1.4.2 Use of Carp AMPs as a Seed Treatment

Bacteria, fungi and viruses not only affect humans and animals, but also are capable of infecting and devastating plant life. Average crop production per annum worldwide is estimated at $950 billion; however, that is only approximately two-thirds of

the potentially attainable $1.5 trillion in crop production (Agrios, 2005). This 36.5% loss is due primarily to the impact of diseases, weeds and insects. Approximately 14.1% of this loss, or $220 billion per year, is lost due to plant diseases (Agrios, 2005; Pimentel, Zuniga, & Morrison, 2005). These losses are not only the actual yield lost, but also the cost of management practices ($0.5 billion), including the development and purchase of pesticides ($36 billion worldwide, $11.2 billion representing 550 million kg of biocides per year in the US alone). Plant pathogens that affect food crops also result in job and income loss in addition to causing widespread hunger and starvation (Agrios, 2005).

There are effective controls for some plant pathogens. Genetic resistance is typically the most desirable form of control, as it is both effective and environmentally friendly; however, not everyone has access to newly developed resistant varieties. Additionally, the response to genetically modified crops and organisms (GMOs) has not been favorable, particularly in Europe, where GMOs are still a matter of great concern. Despite some GMO crops gaining approval for use in the European Union (Directorate General for Health and Consumers as part of the European Commission), many are still banned from being grown in Europe (Commission, 2012). Current control methods for pathogenic organisms include, but are not limited to, chemical applications of fungicides (i.e. foliar, seed and soil applications), heat and radiation treatments, creation of transgenic resistant and control of pathogen vectors. Most current disease management treatments are expensive, have significant side effects (ie. development of resistant pathogens), adversely affect the environment or are currently in the developmental stage and not ready for use (Agrios, 2005)

Seed treatments, such as salt water brines and hot water treatments have been used for over 300 years (Paulsrud, Martin, Babadoost, Malvick, Weinzierl, Lindholm, et al., 2001). In the 1970s, systemic fungicides were introduced as seed treatments, primarily used for the control of loose smut of cereal grains (Agrios, 2005). Over the past 40 years, new seed treatments have been developed, involving advanced fungicides, and the introduction of insecticides and nematicides (Committee, 2007). Seed treatments are currently available for a wide variety of crops to defend against fungi and bacteria. There are some problems related to the use of current seed treatments. Conventional operations use chemical treatments that are often expensive, affect the environment surrounding the seed, and are not effective in treating seed once the pathogen has infected or infested it (McMullen & Lamey, 2010). Availability of seed treatments is limited to those who run organic operations; hot water baths and copper dusts are the most common treatments (Agrios, 2005). Another key issue relating to current seed treatments involves the destruction of the seed itself. Often the level or temperature at which a chemical or water treatment is effective to reduce pathogen load in the seed is enough to kill the embryo as well (Agrios, 2005; Clemens, Jones, & Gilbert, 1977).

Asian Carp FPH could be a possible alternative to current seed treatments as previous research have found FPH enhance seed vigor in soybeans, peas, tomatoes and other crops if used as a seed primer, therefore eliminating the possibility of embryo destruction when treating for pathogenic organisms within the seed (Andarwulan & Shetty, 1999; Horii, McCue, & Shetty, 2007a). The species of the fish, nor the processing parameters, were not identified in either study.

Certain crop diseases have been detrimental to world food supply and continue to be a threat. Table 1 characterizes the plant pathogens my study will examine and their affect on world food supply. Each pathogen is seed borne and are still relevant problems in the world of plant pathology.

1.5 FPH and Seed Vigor

Investigations into the use of FPH on crop growth have been limited but do indicate their to enhance seedling vigor when used as a primer for certain crops (Andarwulan & Shetty, 1999; Horii, McCue, & Shetty, 2007a). Andarwulan and Shetty (1999) used standardized mackerel hydrolysates, but did not specify the processing conditions (ie. enzyme/substrate ratio, degree of hydrolysis, type of enzyme, etc.) used to obtain the hydrolysates. Andarwulan and Shetty (1999) demonstrated that FPH increased both height and weight of the pea plant by 9% and 15%, respectively, as compared to the controls. This was attributed to the increased production of phenolic compounds when seeds were treated with FPH. Phenolic compounds can be a desired crop attribute. Phenolic compounds improve the strength of plant cell walls during the lignin polymerization process of growth (Randhir, Lin, & Shetty, 2004; Randhir & Shetty, 2005). The increase in production of phenolic compounds are also thought to be the cause of enhanced root development and increased height and weight of soybean, tomato and corn seedlings (Horii, McCue, & Shetty, 2007b).

Seed vigor is defined by a series of quality tests conducted on crop seeds based on growth parameters such as germinability and seedling growth under unfavorable conditions and various biochemical aspects (van de Venter, 2000). Due to the fact that

other FPH are typically rich in the amino acid proline, which stimulates auto regulatory functions in plants, FPH are excellent candidates for studying their effects on seedling vigor (Horii, McCue, & Shetty, 2007b; Kristinsson & Rasco, 2000). Another objective in our investigation is to establish a secondary purpose for the application of FPH to seeds. One problem with some current seed treatments used for disease control is their phytotoxic and herbicidal effects (Agarwal & Sinclair, 1997).This is true for even the most simplistic of seed treatments such as organic hot water treatment extensively used in all sectors of agriculture, both in developing and developed countries which if not done correctly can lead to seed injury or death (Maude, 1996; Paulsrud, et al., 2001). Previous studies reported positive effects of FPH as a seed primer on soybeans, corn and tomatoes (Horii, McCue, & Shetty, 2007b).

1.6 Hypothesis ad Research Objectives

1.6.1 Hypothesis

Basic postulations for the outcome of this investigation:

1. Seed vigor in one or more crop plants will be enhanced by the application of fish protein hydrolysates (FPH) as a primer.
2. FHP will exhibit antimicrobial activity toward a range of fungal and bacterial plant pathogens.

1.6.2 Objectives

The objectives of this research include:

1. Determine if Asian carp FPH enhance seed vigor and elicit any beneficial effect when used as a primer.
2. Investigate the antimicrobial properties of FPH derived from Asian carp.
3. Attempt to determine the characteristics of peptides exhibiting antimicrobial effects.
4. Investigate how these fractions affect different causal agents of seed borne diseases.

1.6.3 Implications

Potential implications of this research might include:

1. Possible identification of a novel, natural seed primer for enhance seed vigor
2. Discovery of novel plant disease control compounds
3. Discovery of a secondary product and novel use for an invasive species

Table 1: Microorganisms used in this study.

Name	**Disease**	**Characteristic**
Xanthomonas campestris **pv. *campestris***	Blight and cankers- citrus crops Black rot- Brassicaceae plants	Gram negative bacteria
Rhizoctonia solani	Root, collar, stem rot – numerous plant species (ie. soybean, wheat etc) Foliar blights- cabbage and turfgrass	Fungus
Acidovorax avenae **subsp. *citrulli***	Bacterial fruit blotch (BFB)- watermelon	Gram negative bacteria

CHAPTER 2. ANALYSIS OF SEED VIGOR RESPONSES IN SOYBEAN TO INVASIVE SILVER CARP PROTEIN HYDROLYSATE TREATMENTS[1]

2.1 Abstract

Fish protein hydrolysates (FPH) derived from invasive Silver carp, were used as a seed vigor treatment in soybeans. Soybeans were treated with FPH hydrolyzed for 1, 5.5 and 10 hours with papain (FPH-Pa), pepsin (FPH-P) and trypsin (FPH-T), respectively. Overall vigor tests (accelerated aging and warm and cold germination, dry weight, height, total phenolics and guaiacol peroxidase assessment) were compared to a distilled-water control over a 12-day germination period. Seeds treated with FPH-P and FPH-Pa at 1 hour (23% degree of hydrolysis) elicited the greatest growth responses with increased total percent germination, and higher seedling weights and lengths. GuPx values, indicative of lignification, were also higher in FPH treated soybeans. Results suggest that the use of FPH is beneficial to the stimulation of the proline-linked pentose phosphate pathway, which enhanced the vigor parameters measured.

2.2 Introduction

Seed treatments have been used for centuries for a variety of purposes, including early germination and increased seedling growth and vigor. On-going research has led to

[1] A version of this chapter has been published.
Thomson, S.P., Liceaga, A.M., Applegate, B.M., Martyn, R.D. 2014. Analysis of seed vigor responses in soybean to invasive silver carp protein hydrolysates treatments. *American Journal of Experimental Agriculture.* (DOI: 10.9734/AJEA/2014/13087).

development of treatments that improve seed vigor, which promotes overall heartier plants and increased crop yield (Taylor & Harman, 1990). Many types of seed primers are available, including inorganic salts such as potassium nitrate, and polyethylene glycol (Halmer, 2006; Taylor & Harman, 1990). However, there is still a limited selection of seed primers certified for organic crop producers. Organic primers are essentially limited to hot water treatments and biocontrol agents which can be unstable over relatively short periods of storage and have had varying degrees of success in their ability to promote plant and seed vigor (Groot, 2004; Hermansen, Brodal, & Balvoll, 1999). There is a significant need for organic certified agricultural products, as the demand for organic crops increases. Though the term organic encompasses many regulations and methods of sustainable production as set out by the United States Department of Agriculture (USDA), organic food at its essence is defined as crops and foods that have been produced without use of synthetic inputs (Osteen, Gottlieb, & Vasavada, 2012). Organic food sales have more than doubled since 2004 from an $11 billion dollar industry to $25 billion in 2011 (Osteen, Gottlieb, & Vasavada, 2012). The demand from the public for organic food continues to grow; in 2012 alone, there was a 7.4% increase in organic food sales (Osteen, Gottlieb, & Vasavada, 2012).

Fish protein hydrolysates (FPH) are classified as soluble by-products obtained from proteolytic hydrolysis of crude fish proteins (Mackie, 1982). Production of FPH has attracted attention due to their high nutritive value and improved functionalities arising from the production of short peptides and free amino acids during hydrolysis (Kristinsson & Rasco, 2000). Commercial enzyme preparations are used to maximize FPH yield, with degradation products such as amino acids and peptide fractions varying based on the

structural differences in the proteins and different enzymes used for hydrolysis (Adler-Nissen, 1986; Benjakul, Klomklao, & Simpson, 2009; Worthington & Worthington, 2011). Another important factor affecting the composition of FPH is the degree of hydrolysis (DH) that the native protein undergoes. The DH assists in determining the average peptide chain length and can be controlled by manipulation of the hydrolysis time, enzyme/substrate (E/S) ratio, the type of enzyme being used and the composition of the substrate (fish) (Adler-Nissen, 1986; Benjakul, Klomklao, & Simpson, 2009).

Research on the use of FPH on crop growth is generally limited to uses as in-ground fertilizers, without controlled enzymatic reactions resulting in lower protein quality products that vary greatly in molecular weight. Using standardized mackerel hydrolysates, Andarwulan and Shetty (Andarwulan & Shetty, 1999) demonstrated that FPH increased both the height and weight of pea plants by 9% and 15%, respectively, compared to controls. These increases were attributed to an increase in production of phenolic compounds when seeds were treated with FPH. Phenolic compounds are known to improve the strength of plant cell walls during the lignin polymerization process of growth (Randhir, Lin, & Shetty, 2004; Randhir & Shetty, 2005). The increase in production of phenolic compounds as a result of FPH treatment is also thought to enhance root development and increase the height and weight of soybean, tomato and corn seedlings (Horii, McCue, & Shetty, 2007b). It is important to note, however, that in these studies, the FPH preparation conditions such as enzyme used, enzyme/substrate ratio (E/S), degree of hydrolysis (%DH), and hydrolysis time) were not specified.

In the present study, FPH were produced from silver carp, (*Hypophthalmichthys molitrix*), an underutilized and invasive fish found in the waterways of 18 different

American states, including the Mississippi River System where there is potential for loss of biodiversity resulting from the silver carp colonization (Kolar, Chapman, Courtenay Jr, Housel, Williams, & Jennings, 2005). Silver carp species are now considered "injurious wildlife" by the United States Fish and Wildlife Service (Minnesota DNR, 2005). New recommendations by the Asian Carp Regional Control Committee (ACRCC) for control of silver carp populations primarily relate to the harvest and use of these fish rather than piscicidal options such as chemical and electrochemical barriers (ACRCC, 2012).

The objective of this study was to produce FPH from invasive silver carp under controlled hydrolysis conditions (DH, E/S ratio, enzyme used), and to examine the effects of the FPH on soybean seed vigor, using standard vigor tests (accelerated aging, germination and cold germination, weight, height, total phenolics and guaiacol peroxidase assessment). The ultimate goal is to be able to use these fish to develop an economic agricultural product to improve seed vigor for organic crop producers while aiding in the control of an invasive fish species.

2.3 Materials and Methods

2.3.1 FPH preparation

Fresh silver carp (*Hypophthalmichthys molitrix*) were harvested from the Wabash River (Lafayette, IN, USA). The fish were transported on ice within 24 h to the Purdue University Food Science Department where they were beheaded, eviscerated, filleted and immediately frozen at -20 °C for later use. FPH were produced as described by Liceaga-Gesualdo and Li-Chan (1999) with some modifications. The silver carp fillets were thawed, and mixed with water (1:2 w/v fish-distilled water), then homogenized in a

commercial blender (Waring Heavy Duty Blender 4L, Sigma-Aldrich, St. Louis, MO, USA) at 18000 rpm for 3 min. The enzymes pepsin (E.C.3.4.23.1), papain (E.C. 3.4.22.2) and trypsin (E.C. 3.4.21.4) (Sigma-Aldrich, St. Louis, MO, USA) were added separately to the slurries at 2.5% (w/w) of the protein content in the fish. Each mixture was agitated in a water bath maintained at 55°C and underwent hydrolysis for 1.0, 5.5 and 10 hours. After hydrolysis, samples were pasteurized (85°C for 15 min) to inactivate the enzyme and centrifuged at 15000g for 15 min and 4°C (Avanti J-26S Centrifuge, Beckman-Coulter INC. CA, USA) to separate the insoluble fraction. The FPH supernatant was collected from each enzyme treatment [pepsin (FPH-P), trypsin (FPH-T) and papain (FPH-Pa)] and stored at -80°C until use. A FPH control (FPH-C) was prepared using the same process conditions as above except that the fish slurry was immediately pasteurized without enzyme addition.

The best performing FPH elicitors as determined from preliminary seed vigor trials (data not shown) were chosen for subsequent trials.

2.3.2 Degree of hydrolysis (DH)

The DH was calculated using the trinitrobenzene sulfonic acid (TNBS) method described by Adler-Nissen (1986) with modifications by Liceaga and Li-Chan (1999). Briefly, 10 mL aliquots from each trial were mixed with 10 mL of 24% trichloroacetic acid (TCA) and centrifuged for 5 min at 12100g. Aliquots (0.2 mL) from the supernatant were mixed with 2mL of sodium borate buffer (0.2 M at pH 9.2) and 1.0 mL TNBS (4.0 mM) and then incubated for 30 min in the dark at room temperature. This was followed by the addition of 1 mL of 2.0 M NaH_2PO_4 containing 18 mM Na_2SO_3. The absorbance

was read at 420 nm using a UV-Vis spectrophotometer (Beckman-Coulter DU640 UV-Vis Spectrophotometer, Beckman-Coulter Inc. CA, USA). DH was calculated using the equation:

$$\%DH = h/h_{tot} \times 100$$

where h represents the number of peptide bonds broken and h_{tot} represents the total number of peptide bonds present. In fish protein concentrate, h_{tot} was assumed to be 8.6 meq/g (Adler-Nissen, 1986).

2.3.3 SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on FPH-P, FPH-Pa and FPH-T. To 40μL of each FPH sample, 2 μL 2-mercaptoethanol, 20 μL 10% SDS, and 5 μL tracking dye (0.05% bromophenol blue) in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0 was added. Samples were heated in a boiling water bath for 10 min before being centrifuged at 10000g for 5 min. Electrophoresis was performed using a PhastSystem™ (G.E. Healthcare Biosciences, Pittsburgh, PA, USA) with 10-15% gradient polyacrylamide PhastGels. After electrophoresis, the gels were stained for 45 min with Coomassie blue (0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid) and destained with 30% methanol and 10% acetic acid until bands were visible. To preserve the gels, gels were placed in a solution of 10% acetic acid and 5% glycerol. The approximate molecular weights were determined using high range molecular weight standards (Sigma-Aldrich, St. Louis, MO, USA).

2.3.4 Free amino acid composition

Free amino acid composition analysis of FPH samples was completed by Bindley Discovery Park Proteomics Facility at Purdue University using a Beckman HPLC (126 pump, 166 detector, 507 autosampler) with a Waters AccQ Tag amino acid analysis column and UV detector, calibrated with amino acid standards (Pierce, Rockford, IL).

2.3.5 Seed treatment

Seed treatments and preparation of plant materials were in accordance with methods modified from those described by Randhir and Shetty (2005). Seeds of the soybean (*Glycine max)* variety Viking 2265 (Johnny's Seeds, Waterville, ME) were used for this study. Seeds were washed using a 1:5 ratio of bleach (5.25% v/v sodium hypochlorite) to distilled water to remove possible contaminants and then rinsed twice for 1 min each with distilled water to remove excess bleach. Decontaminated seeds were placed into 100 mL of treatment solution. Each treatment solution contained 5 mL/L (v/v) FPH supernatant to distilled water, containing 107.6 ug/mL of protein per FPH treatment solution. The primed seeds were incubated for 24 hours in an orbital shaker set at 120 rpm and excess water was drained. This method of seed treatment and priming was used for all vigor assessment trials.

2.3.6 In-Vitro Soybean Seed Germination

A total of 50 seeds per FPH treatment were placed in individual treatment solutions of 5 mL/L (v/v) FPH supernatant to distilled water for 24 hours. Treated seeds were then placed in Petri dishes lined with filter paper and covered with aluminum foil to block

light during germination. Seeds were incubated at room temperature (~22°C) and sprayed four times daily with sterile distilled water to maintain high moisture. Seeds primed only with distilled water were used as controls. Germination data was collected on day 4, 8 and 12. There were three replicate trials per treatment for a total of 150 seeds per treatment.

2.3.7 Growth Chamber Seed Germination

A total of 50 soybean seeds per FPH treatment were placed in sterile potting soil, approximately (2.5 cm) deep and allowed to germinate in a growth chamber over a 12-day period. Seedlings were evaluated on day 4, 8 and day 12 for fresh weight (including roots), length of seedling (from root to shoot) and root to shoot length ratio. All tests were completed in duplicate. Temperatures in the growth chamber were maintained at 30^{o}C during the day and 20^{o}C at night. Relative humidity was maintained at 70% RH.

2.3.8 Warm seed germination

Percent germination of FPH-treated seeds was conducted using the ragdoll method, as outlined by the United States Department of Agriculture (Douglas, Grabowski, & Daughtry, 2003). Treated soybeans (triplicate trials with 50 seeds per treatment and trial) were spread out along the center of two sterile paper towels. The paper towels were moistened with sterile water and then tightly rolled into tubes. The tubes were placed in plastic bags and incubated for 4 days at 30^{o}C. Germination was defined as emergence of the radical. Percent germination was calculated as the number of seeds germinated versus the total number of seeds.

2.3.9 Cold Seed Germination

A modified 'shoebox method' for cold germination as used by the Indiana Crop Improvement Association (ICIA) and the Oregon State University Seed Laboratory and in accordance with methods developed by the Association of Official Seed Analysts (AOSA) was used to test seed vigor under cold stress conditions (Association of Official Seed Analysts, 1983; Indiana Crop Improvement Association, 2014; Oregon State University, 2014). Soybean seeds were treated with FPH solutions as was used for in-vitro seedling testing. After draining excess water, seeds were placed on sterile paper towels and dried at 30^{o}C for 16 hours. Seeds were then placed in 2.5 cm of sterile vermiculite moistened to a 75% saturation level and stored at 10^{o}C for 7 days. On day 8, the temperature in the growth chamber was increased to 25^{o}C, and maintained for an additional 5 days. Percent seed germination was determined on day 12.

2.3.10 Accelerated aging test

The accelerated aging test described by the Iowa State University Seed Testing Laboratory in accordance with methods established by the International Seed Testing Association (ISTA) and AOSA was used (Association of Official Seed Analysts, 1983; Indiana Crop Improvement Association, 2014; Iowa State University, 2014). Both control and FPH-treated seeds were placed on sterile paper towels and dried at 30°C for 16 hours. Seeds were then placed in trays elevated above 40 mL of water in a sealed box. The boxes were then placed in a growth chamber and exposed to high temperature (45^{o}C) and high moisture (approx. 100% relative humidity) for 72 hours. The seeds were then

subjected to the warm germination protocol previously described. Percent germination was determined after 4 days.

2.3.11 Total phenolics assay

Total phenolic content was quantified using the Folin-Ciocalteau reagent as outlined by Andarwulan and Shetty (1999). Sprout and leaf tissue from the seedlings were extracted using 99.5% ethanol (1ml ethanol/50 mg fresh weight) and distilled water. The tissue was homogenized using a mortar and pestle and centrifuged for 10 min at 17000g. One mL of supernatant was added to 0.5 mL of Folin-Ciocalteau reagent, 1mL of 99.5% ethanol and 5 mL distilled water and incubated for 5 min at room temperature (approximately 22°C). After incubation, 1 mL of 5% Na_2CO_3 (w/v) was added to each sample. The resulting solution was vortexed and incubated in the dark at room temperature (~22°C) for 60 min. Absorbance was read at 725 nm using a UV-Vis spectrophotometer. Total phenolics was calculated as gallic acid equivalent (GAE) using the equation:

$$T = C \times (V/M) \quad [2]$$

where T represents the total phenolic content in mg/g, C is the concentration of gallic acid as established by the gallic acid calibration curve in mg/mL, V is the volume of leaf extract used in mL and M is weight of the leaf extract in g.

2.3.12 Preparation of enzyme and protein extracts

Protein was extracted based on modifications to the protocol by Horii et al. (2007b). Tissue (100 mg) derived from the shoots of soybean was placed in 2 mL of the enzyme extraction buffer containing 0.5% (w/v) polyvinylpyrrolidone, 3mM EDTA and 0.1 M potassium phosphate buffer and ground using a chilled mortar and pestle. The tissue-buffer mix was centrifuged for 10 min at 6000g and held at 4°C. The supernatant was then used for the protein and guaiacol peroxidase (GuPX) assays.

2.3.13 Guaiacol peroxidase (GuPX) assay

GuPX was determined as described by McCue et al. (2000). Supernatant (50 uL) from the extraction buffer / tissue homogenate solution was placed into a GuPX assay solution containing 0.1 M potassium phosphate buffer (pH 6.8), 20 mM guaiacol solution and 0.2 mM hydrogen peroxide. Total volume was 1 mL. The increase in absorbance at 470 nm was recorded over a period of 5 minutes and indicated the production of tetraguiaicol. The GuPX solution without the tissue homogenate was used as a blank. GuPX activity was reported as nmol tetraguaiacol/min/mg protein.

2.3.14 Protein Determination

Protein content of soybean tissue was determined by the bicinchoninic acid protein assay (BCA) according to manufacturer's protocol using bovine serum albumin as a standard (Thermo Scientific Pierce Pierce Biotechnology, Rockford IL). Protein content was used for the calculation of GuPX activity.

2.3.15 Statistical analysis

All data were subjected to an analysis of variance (ANOVA) using Minitab® 16 software (Minitab ®, PA, USA) to determine whether the treatments had a significant effect on seed germination and vigor. The difference between treatments was analyzed by Fisher's test with statistical significance at $\alpha = 0.05$ unless otherwise noted.

2.4 Results and Discussion

Our study sought to further demonstrate the ability of FPH to elicit phenolic responses in seeds during conditions of stress (Randhir, Kwon, & Shetty, 2009) and determine which treatments used to produce the FPH were most beneficial.

After 1 hour of hydrolysis, pepsin was able to hydrolyze the crude fish protein to a DH of 23.3% (Table 2) and increasing the hydrolysis time to 5.5 hours had no additional increase on the DH (23.5%). Trypsin hydrolyzed the proteins to a higher DH (26.4%) than pepsin after 5.5 hours with a slight increase in DH after 10 hours (27.5%). Papain hydrolyzed the fish protein more extensively than either pepsin or trypsin, yielding 23.8% DH after 1 hour and 36.3% DH after 10 hours. These results are consistent with findings by Ng and Khan (2012) who reported that pepsin and trypsin had similar DH when hydrolyzing palm kernel expeller proteins. By using different enzymes and varying hydrolysis times, we produced hydrolysates containing different peptide fractions. These differences contributed to the variance observed in seedling vigor responses. The differences in substrate preferences of the enzymes used in protein hydrolysis resulted in distinct hydrolysates that stimulated plant growth unique to the individual hydrolysates.

As illustrated by the SDS-PAGE profiles, FPH-P hydrolysed for 1 hour, showed 2 clear bands in the 200 and 66 kDa range (Figure 1, lane 2). As hydrolysis continued, these bands dissipated into the lower molecular weight range (lanes 3 and 4). In contrast, FPH-Pa yielded only low molecular weight peptides (lanes 5, 6, 7) even at 1 hour of hydrolysis, which is consistent with the biochemical nature of papain, which hydrolyzes a broader spectrum of protein bonds compared to both trypsin and pepsin (Worthington & Worthington, 2011). In the case of FPH-T, separation of peptides was similar for all hydrolysis times; banding occurred at 66 kDa, 200 kDa and 116 kDa for all three trypsin hydrolysis times (data not shown). Dense banding at lower molecular weight markers occurred in FPH-T hydrolyzed for 10 hours confirming the presence of smaller peptides in proteins hydrolyzed for longer periods of time.

In order for plants to synthesize proteins necessary for growth, essential amino acids must be readily available (Ardebili, Moghadam, Ardebili, & Pashaie, 2012). During hydrolysis, the original protein source is fractionated into smaller peptides and free amino acids (Adler-Nissen, 1986), which increases the amount of ammonia and soluble nitrogen compared to the original native protein (Apiwatanapiwat, Vaithanomsat, Somkliang, & Malapant, 2009; Liaset, Nortvedt, Lied, & Espe, 2002; Veselá & Friedrich, 2009). Nitrogen is used extensively as a plant fertilizer and the increased availability of nitrogen from the FPH to the plant cell for synthesis of essential amino acids likely would lead to increased seedling vigor.

Previous studies have shown that foliar applications of amino acids increases plant growth parameters such as number of leaves, fruits, height of plant and root structure (Abdel-Mawgoud, El-Bassiouny, Ghoname, & Abou-Hussein, 2011; Ardebili,

Moghadam, Ardebili, & Pashaie, 2012; Horii, McCue, & Shetty, 2007b; Randhir, Kwon, & Shetty, 2009; Randhir, Lin, & Shetty, 2004). The amino acid proline specifically has been shown to alleviate abiotic stressors during plant growth (Randhir & Shetty, 2005). Hydrophilic amino acids such as lysine (LYS) and asparagine (ASP) (Appendix A) are more easily absorbed into plant cells than peptides due to their smaller size and the differences in polarity and hydrophobicity of peptide fractions (Stacey, Koh, Granger, & Becker, 2002). These amino acids also increase the concentration of osmo-regulatory components in plant cells (Abdel-Mawgoud, El-Bassiouny, Ghoname, & Abou-Hussein, 2011). In our study the increased availability of free amino acids as a result of hydrolysis elicited a greater growth response as compared to water controls.

Elicitors such as FPH have been documented to stimulate plant metabolic pathways when applied to seeds as priming agents (Randhir & Shetty, 2005). Previous research has shown that the proline-linked pentose phosphate pathway (PPP) is stimulated predominantly by the high proline and glutamic acid content of the FPH, and therefore likely responsible for the increase in plant phenolic content (Randhir, Kwon, & Shetty, 2009). Previous studies also show that when plants are placed under stress situations, as we have done by germinating our seedlings for longer periods of time in the dark with the addition of an elicitor, synthesis of phenolic compounds increases ((Randhir, Kwon, & Shetty, 2009). Phenolics may serve to protect plants in situations in which they have been stressed by producing antioxidants with reactive oxygen species (ROS) scavenging capabilities (Randhir & Shetty, 2005). Simple phenolic compounds, as measured by GuPX activity, are also important in the lignification of cell walls (Cakmak & Horst, 1991).

On day 4, seeds treated with FPH-P at 1 hour of hydrolysis produced higher totals of phenolic compounds (1.27 mg GAE/g FW) than the water control (0.59 mg GAE/g FW) (Figure 2). The necessity for cellular protection via increased lignification of cell walls is increased in stressful environments, which the seedlings were exposed to during dark germination, such as in the accelerated aging and cold germination trials. This is reflected by the higher percent germination in the accelerated aging test for seeds treated with FPH-P at 1 hour of hydrolysis (42.6%) as compared to 30.3% for water controls which correlated with the higher phenolic content of FPH-P at 1 hour of hydrolysis at day 4 (Figure 2). Accelerated aging of soybeans and other oil seeds has been found to increase lipid peroxidation and accelerate damage due to free radicals (Sung, 1996). The increased total phenolic content, but lower GuPX content (Figure 5) of the treated seeds suggests that the pentose-phosphate pathway was stimulated and phenolic compounds present in the seed were diverted principally to increasing ROS antioxidant and defense activities of the cells on day 4. This in turn may enhance vigor during stress caused by heat and excessive humidity as in the situation of our accelerated aging test. Stimulation of the pentose-phosphate pathway was also likely increased in the seedlings during the warm germination trials (Table 2), as percent germination was higher for both FPH-P at 1 hour (91.0%) and FPH-Pa at 1 hour (85.8%) compared to water controls (72.7%). Seedlings treated with both of these FPH treatments also had the highest phenolic content on day 4 (Figure 2).

Total phenolic content decreased on day 8 in seeds treated with FPH-P at 1 hour, FPH-Pa at 1 hour and FPH-T at 10 hours. Other FPH treatments and the water control had consistent increases in phenolic content. GuPx concentration, however, decreased on

day 8 for all treatments (Figure 3-4) with the exception of FPH-P at 1 hour of hydrolysis (Figure 5), also suggesting that the cells might be using the phenolic compounds as antioxidants. Despite decreases in GuPX content, FPH-Pa at 1 hour showed longer seedlings at both day 8 (30 mm) and day 12 (53 mm) germination times as seen in Figure 6 compared to the 12-day water control (28 mm total = on day 8, 46.8 mm total length on day 12). Additionally, FPH-Pa at 1 hour produced heavier seedlings (Figure 7) on day 12 (1.38 g/seedling) compared to water controls (1.25g/seedling on day 12). Both weight and length values reflect a larger GuPX value for FPH-Pa at 1 hour of hydrolysis on both day 8 and day 12 compared to water control. Overall, soybeans treated with FPH consistently had higher GuPX values on day 8 and day 12. Seeds treated with FPH-P at 1 hour of hydrolysis had increased GuPX content over the entire germination period, which is consistent with an increase in the pentose phosphate pathway and the generation of ROS antioxidant defenses to peroxidation in the initial stages of germination. However, exhaustion of these phenolics by day 8 might lead to a more extensive use of the compounds for growth and lignification (Horii, McCue, & Shetty, 2007a).

After 12 days of germination, both total phenolics and GuPX content increased for all treatments (Figure 3-5), which could reflect increased cellular synthesis of phenolic compounds and metabolic efforts being diverted to lignification and plant growth (Horii, McCue, & Shetty, 2007b; Randhir, Kwon, & Shetty, 2009; Randhir, Lin, & Shetty, 2004). No differences were found between phenolic contents for treatments on day 8 or day 12; however, FPH-P at 1 hour did have a higher phenolic content on day 12 (1.43 mg GAE/g FW compared to 1.10 mg GAE/g FW for the water control).

In terms of warm germination, no significant differences were found in final germination percentages between soybean seeds treated with FPH or water when subjected to cold stress (Table 2). FPH-T at 5.5 hours of hydrolysis (73.0% germination) appeared to increase the percent germination compared to other treatments and the water control (61.0% germination), however it was not statistically different. Cold stress decreased the overall germination for all treatments. Cold stress elicits different metabolic responses from those caused by heat stress and dark stress (Heidarvand & Amiri, 2010). Plants affected by cold stress use calcium-gated channels to mediate the expression of the hormone abscisic acid (ABA). ABA further regulates genes involved with physiological adaptations to the cold, such as delayed embryogenesis and closure of stomata to minimize fluid losses (Sung, 1996). The proline-linked pentose phosphate pathway (PPP) does not come into effect during periods of cold or drought stress (Heidarvand & Amiri, 2010), therefore the FPH do not have the same effect on the metabolic pathways regulating cold stress.

There does not appear to be a clearly defined trend in regards to which hydrolysis treatments stimulated growth most effectively. FPH-P at 1 hour of hydrolysis appears to be the most effective at stimulating the PPP. Pepsin has broad specificity, preferentially cleaving aromatic or carboxylic L amino acids, or at the C terminal of phenylalanine, leucine or glutamic acid (Worthington & Worthington, 2011). FPH-P at 1 hour of hydrolysis contained free proline (7.08 g/100g protein), glutamine (3.92 g/100g protein protein) and glutamic acid (4.55 g/100g protein) (Appendix A). Glutamic acid and glutamine have been shown to improve seedling vigor and elicit responses from the PPP and therefore could be responsible for the improvement of seedling vigor in this study

(Randhir, Kwon, & Shetty, 2009). FPH-Pa at 1 hour of hydrolysis was also able to improve some aspects of seedling vigor. Papain has a large active site and a lack of defined specificity (Worthington & Worthington, 2011). Amino acids such as proline, glutamine and glutamic acid were released during hydrolysis with papain, aiding in elicitation of the PPP and improving overall vigor; FPH-Pa at 1 hour of hydrolysis contained 5.55 g, 5.20 g and 1.22 g/100g protein of free proline, glutamine and glutamic acid, respectively. On the other hand, seedling vigor was improved only minimally when soybean seeds were treated with FPH-T. Trypsin is substrate specific, and does not cleave near the amino acid proline (Worthington & Worthington, 2011). This is a detriment to the efficacy of FPH-T to stimulate the PPP, as proline is integral in the function of this metabolic pathway ((Randhir & Shetty, 2005). FPH-T treatments contained less free proline, than other treatments, but contained more free glutamine (Appendix A).

2.5 Conclusion

Protein hydrolysates derived from silver carp stimulated growth of soybean seedlings when applied as a seed primer. FPH created both pepsin (23.3% DH) and papain (23. 4% DH) led to shorted peptides and released free proline, glutamine and glutamic acid. This resulted in the stimulation of PPP, which increased phenolics responsible for protection against peroxidation. FPH-P and FPH-Pa, hydrolyzed for shorter periods of time (ie. 1 hour) at an E/S ratio of 2.5% have an ideal free amino acid and peptide composition for the overall stimulation of seedling vigor. With controlled-hydrolysis FPH have potential to be used as organic seed treatments and as an economically feasible value-added product that aids in controlling an invasive fish

species. Further studies will be required to investigate the stability of FPH for practical applications.

Table 2: Average percent germination[a] of seedlings by treatment.

	Average percent germination (%) per treatment by vigor test				
Treatment[b]	Hydrolysis Time (hours)	DH (%)	Warm Germination	Cold Germination	Accelerated Aging
FPH-P	1	*23.3*	91.0 (4.4)^{A}	47 (43.8)^{A}	42.6 (31.9) ^{A}
	5.5	*23.5*	80.8(11.3)^{AB}	49(21.2) ^{A}	28.6 (0.96)^{BC}
FPH-T	5.5	*26.4*	88.8 (4.5)^{A}	73 (7.1) ^{A}	25.8 (5.3) ^{BC}
	10	*27.5*	89.9 (4.0)^{A}	64 (8.5) ^{A}	36.1 (1.5) ^{BC}
FPH-Pa	1	*23.8*	85.8 (1.8)^{A}	64 (19.8) ^{A}	22.5 (7.1) ^{C}
	10	*36.3*	85.7 (9.3)^{A}	67 (4.2) ^{A}	44.9 (20.0)^{AB}
Water Control			72.7 (2.3)^{B}	61 (18.4) ^{A}	30.3 (5.18)^{BC}

[a] Values in parentheses represent the standard deviation (n=3) associated with each data set. Data bearing different uppercase letters (A, B, C) indicate significantly different ($P < 0.05$).

[b] FPH-P = pepsin hydrolyzed proteins, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed proteins, Water control = seeds soaked in distilled water.

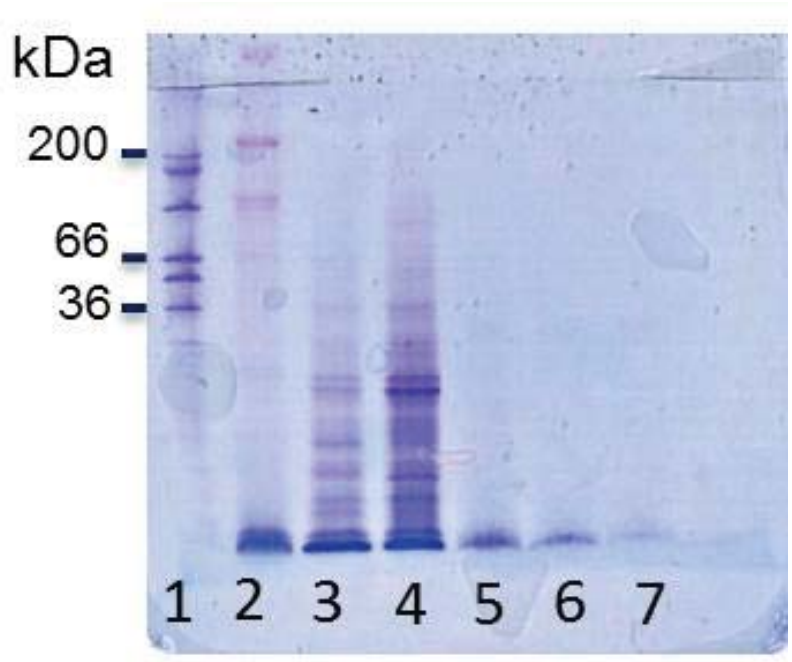

Figure 1. SDS-PAGE profiles of FPH-P (lanes 2, 3, 4) and FPH-Pa (lanes 5, 6, 7) for 1, 5 and 10 hours, respectively. Wide range molecular weight protein standards (lane 1) representing 36 to 200 kDa.

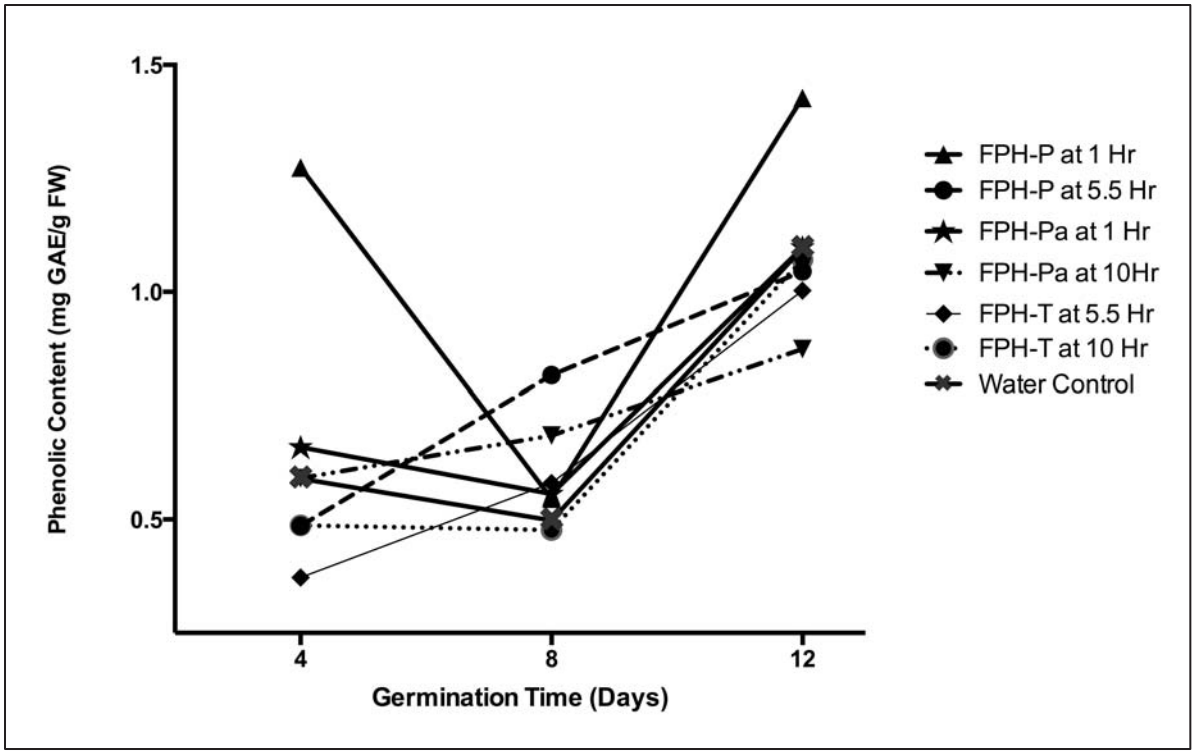

Figure 2. Effect of FPH treatments on total phenolic content in mg Gallic Acid Equivalents (GAE) of soybean seedlings as compared to water primed seedlings: **(a)** FPH-P **(b)** FPH-T **(c)** FPH-Pa. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

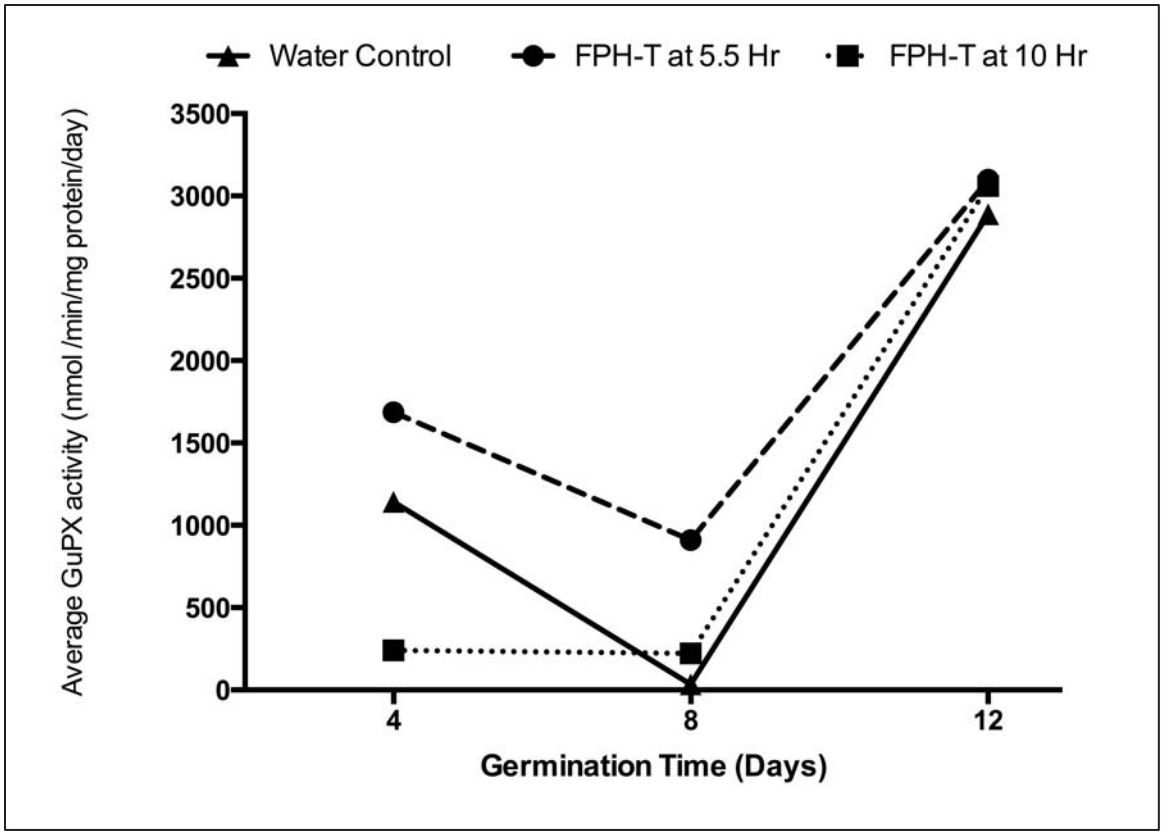

Figure 3. Effect of FPH treatments on Guaiacol Peroxidase Activity in nmol tetraguaiacol per minute per mg of protein of FPH-T treated soybean seedlings as compared to water primed seedlings. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

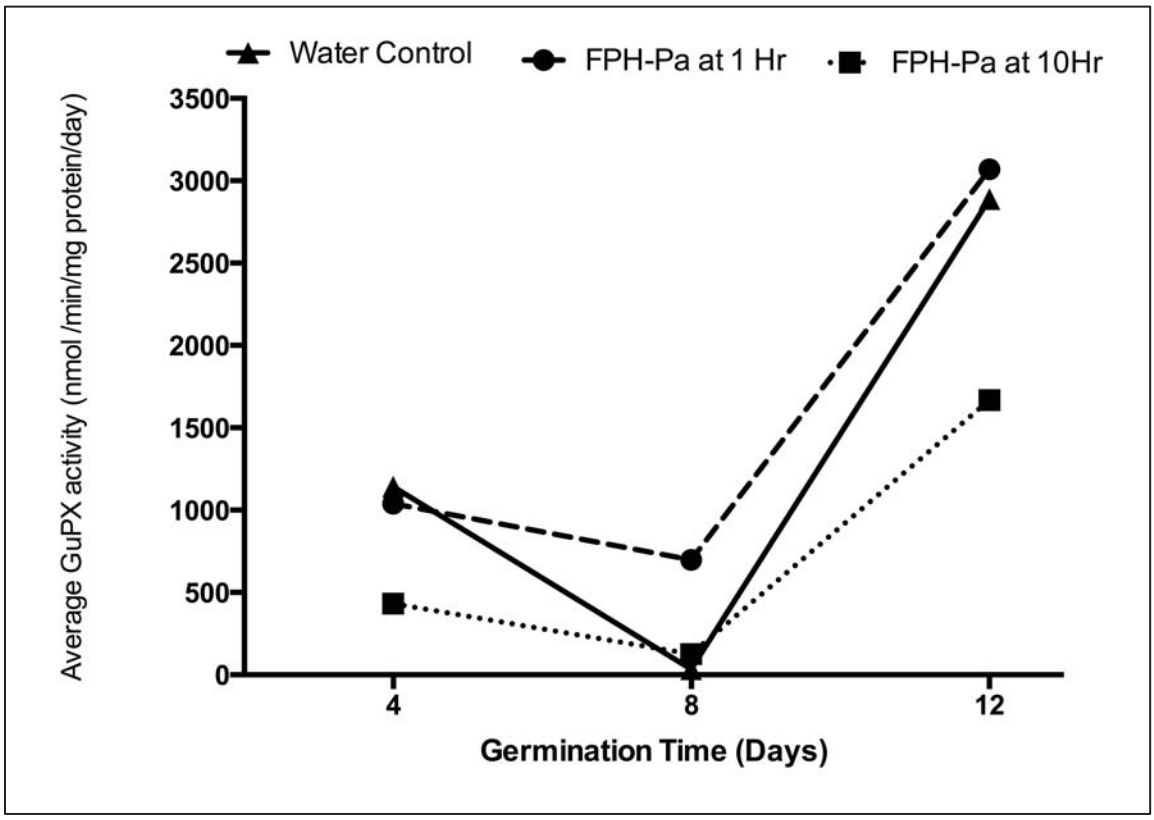

Figure 4. Effect of FPH treatments on Guaiacol Peroxidase Activity in nmol tetraguaiacol per minute per mg of protein of FPH-Pa treated soybean seedlings as compared to water primed seedlings. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

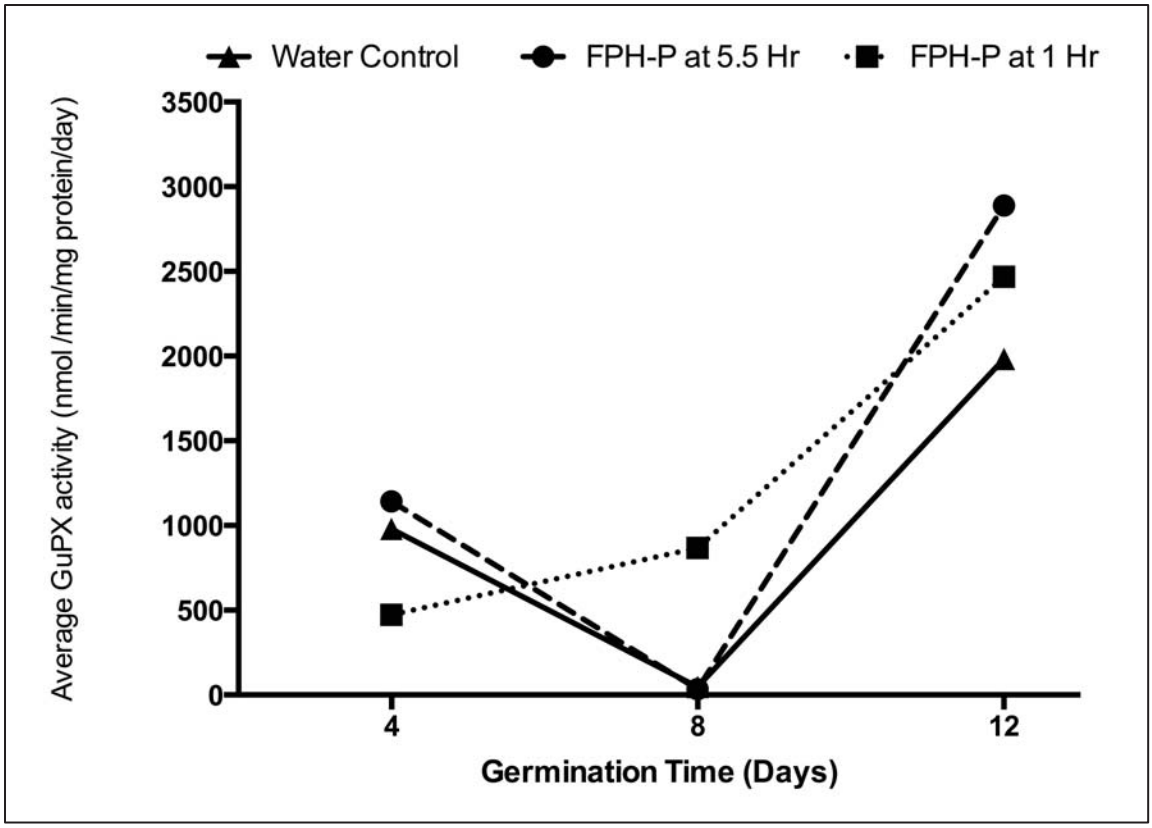

Figure 5. Effect of FPH treatments on Guaiacol Peroxidase Activity in nmol tetraguaiacol per minute per mg of protein of FPH-P treated soybean seedlings as compared to water primed seedlings. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

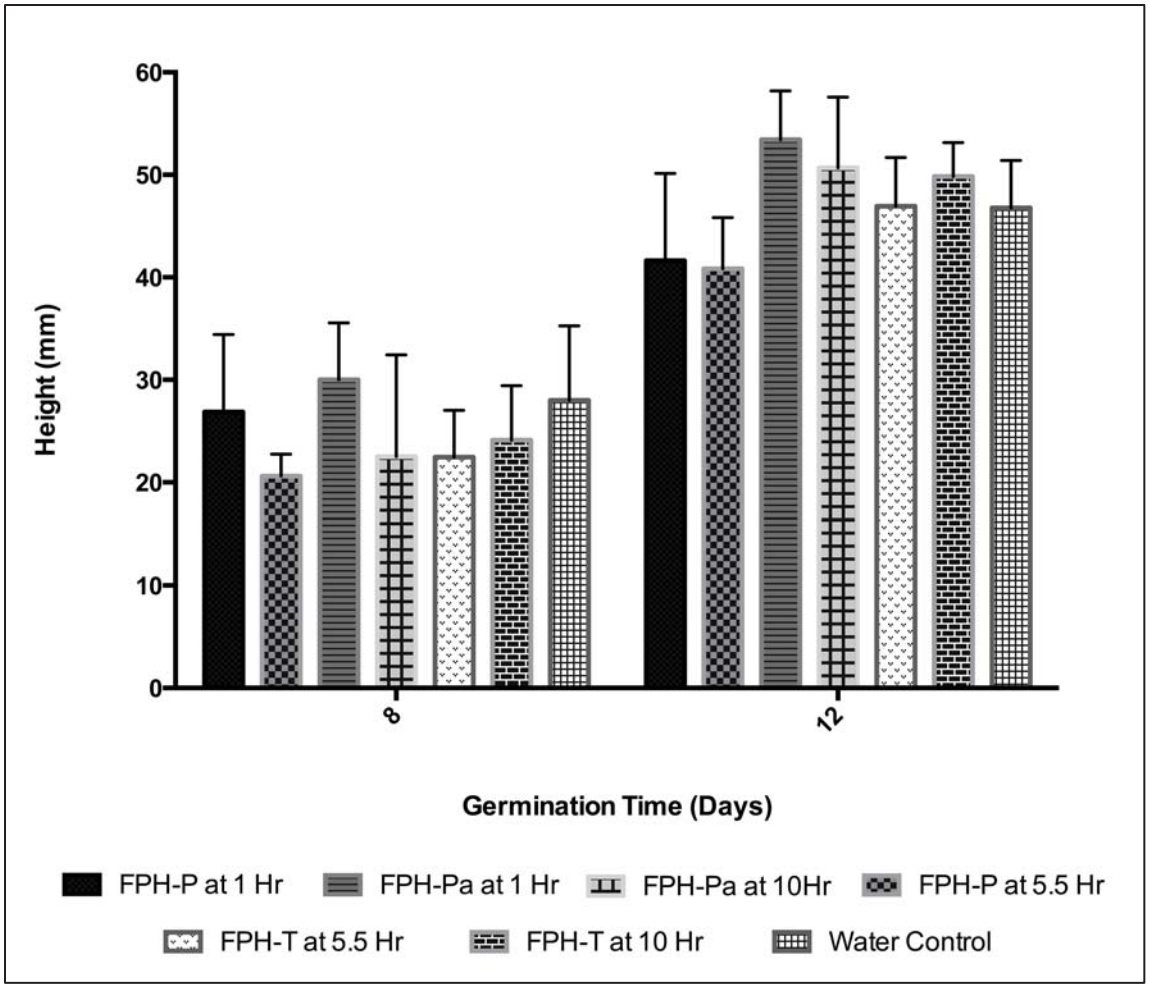

Figure 6. Effect of FPH treatments and FPH-C on soybean seedling lengths as compared to water control seedlings. Bars represent average values +/- SD: **(a)** FPH-T **(b)** FPH-P **(c)** FPH-Pa. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

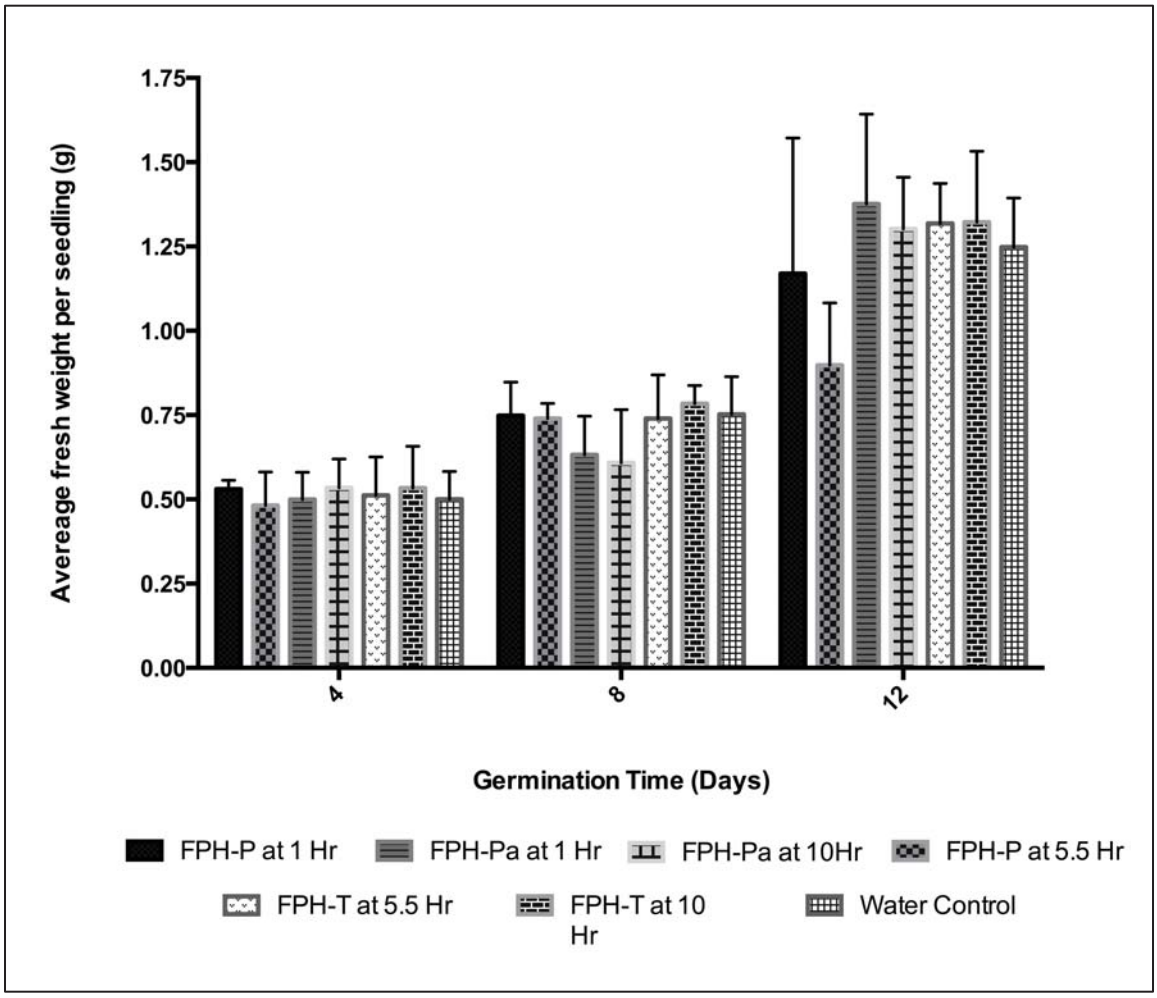

Figure 7. Effect of FPH treatments and FPH-C on soybean seedling weights as compared to water control seedlings. Bars represent average values +/- SD: **(a)** FPH-T **(b)** FPH-P **(c)** FPH-Pa. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

CHAPTER 3. ANALYSIS OF SEED VIGOR RESPONSES IN WATERMELON TO INVASIVE SILVER CARP PROTEIN HYDROLYSATE TREATMENTS

3.1 Abstract

The efficacy of fish protein hydrolysates (FPH) as seed treatments for stimulation of watermelon growth was examined. FPH treatments were created using invasive silver carp protein and hydrolyzed with the enzymes trypsin (FPH-T), pepsin (FPH-P) and papain (FPH-Pa), for 1, 5.5 or 10 hours. Vigor parameters tested included seedling weight, length, total phenolics, accelerated aging, cold germination and warm germination. FPH treatments increased all parameters with the exception of total phenolic content as compared to water controls, indicating the proline-linked phosphate pathway was not stimulated. Differences in amino acid composition between FPH treatments have an effect on growth. The amino acids TRP, MET, PRO, TYR, THR, VAL, LYS and ASP may play a key role in growth stimulation.

3.2 Introduction

Seed treatments have been used extensively for hundreds of years to prevent against seed borne diseases and improve germination (Du Toit, Derie, Brissey, Holmes, & Gatch, 2009). The use of fish protein hydrolysates (FPH) as a seed treatment has previously been successful in peas, soybean, tomatoes and corn to improve plant growth and seedling vigor (Andarwulan & Shetty, 1999; Horii, McCue, & Shetty, 2007b).

Currently, seed treatments are limited to chemical options and ineffective natural seed treatments (Du Toit, Derie, Brissey, Holmes, & Gatch, 2009; Hermansen, Brodal, & Balvoll, 1999). FPH are potential natural, organic alternatives to what is presently available. FPH used in this study was derived from silver carp, an invasive fish species residing in the Mississippi River System (Asian Carp Regional Coordinating Committee, 2012).

Watermelon is one of the most consumed melons in the United States. The State of Indiana ranks among the top five in the production of specialty crops, in particular watermelon, cantaloupes and tomatoes. In particular, Indiana is ranked for fresh market watermelon production in the United States (Marshall, Alexander, Dennis, Lopez, Quagrainie, 2008).

The objective of this study was to determine the efficacy of FPH derived from invasive silver carp on the growth and vigor of watermelon seedlings. Vigor tests included accelerated aging tests, germination, cold germination, weight determination, height determination and total phenolic content assessment. In this study, characterization of the FPH including preparation conditions, free amino acid composition and approximate peptide mass are identified in order to determine characteristics of the FPH that could be potentially stimulating growth.

3.3 Materials and Methods

3.3.1 FPH Preparation

Silver carp meat was hydrolyzed using the enzymes papain, pepsin and trypsin and hydrolyzed for 1, 5.5 or 10 hours. The treatments, papain hydrolyzed protein (FPH-Pa), pepsin hydrolyzed protein (FPH-P) and trypsin hydrolyzed protein (FPH-T) were prepared according to LiceagaGesualdo & LiChan (1999) with modifications as described in section 2.3.1. The FPH treatments best able to elicit vigor responses from preliminary seed vigor trials (data not shown) were chosen for further testing.

3.3.2 Degree of Hydrolysis (DH)

The DH of the FPH samples was determined based on the trinitrobenzene sulfonic acid (TNBS) method as described by Adler-Nissen (1986) with modifications as described in section 2.3.2.

3.3.3 SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on all treatments (FPH-T, FPH-Pa, FPH-P at 1, 5.5 or 10 hours of hydrolysis), as described in section 2.3.3. The molecular weights of FPH-T and FPH-P were compared to high range molecular weight standards, whereas FPH-Pa treatments were compared to ultra-low molecular weight standards (Sigma-Aldrich, St. Louis, MO, USA).

3.3.4 Free amino acid characterization

The free amino acid content was characterized and quantified for each FPH treatment. This was performed by Bindley Discovery Park Proteomics at Purdue University (West Lafayette, IN, USA). A Beckman high performance liquid chromatograph (126 pump, 166 detector, 507 autosampler HPLC) with a Waters AccQ Tag amino acid column and UV detector, calibrated with amino acid standards (Pierce, Rockford, IL, USA) was used to perform the assay.

3.3.5 Seed treatment

Preparation of the treatment solutions and priming of the seeds was conducted according to methods outlined by Randir and Shetty (2004) with some modifications as described in section 2.3.5. Seeds of untreated watermelon (*Citrullus lanatus*) variety Crimson Sweet (Harris Seeds, Rochester, NY, USA) were used for this study.

3.3.6 In-Vitro seed germination

A total of 25 seeds per FPH treatment were treated according to methods outlined in section 2.3.6. Seeds primed with sterile distilled water were used as a control. Seeds were incubated in the dark at room temperature (~25^{o}C) and sprayed 10 times daily with sterile distilled water to maintain a high moisture environment. Root and shoot length, seedling weight (dry) and total phenolics were evaluated on day 12. Three replicates trials per treatment were completed.

3.3.7 Warm seed germination

Percent germination was conducted according to the ragdoll method, as outlined by the United States Department of Agriculture (Douglas, Grabowski, & Daughtry, 2003) with modifications, as described in section 2.3.8. One hundred seeds per treatment were spread out along the center of a moistened, sterile paper towel. The paper towels were then rolled tightly into tubes, placed into plastic bags and incubated for 12 days at 35^{o}C. Moisture was maintained by spraying towels every second day with sterile distilled water. Trials were completed in duplicate.

3.3.8 Cold seed germination

Methods described by the Indiana Crop Improvement Association (ICIA) and the Oregon State University Seed Laboratory, in accordance with the standards outlined by the Association of Official Seed Analysts (AOSA) were used to test the efficacy of the FPH treatment in mitigating cold stress in the watermelon (Association of Official Seed Analysts, 1983; Indiana Crop Improvement Association, 2014; Oregon State University Seed Laboratory, 2014). Methods are described in section 2.3.9. Total percent germination was evaluated after 12 days.

3.3.9 Accelerated aging test

The accelerated aging test was performed according to methods described by the Iowa State University Seed Testing Laboratory, in accordance to the standards identified by the International Seed Testing Association (ISTA) and the AOSA (Association of Official Seed Analysts, 1983; Iowa State University Seed Testing Laboratory, 2014. Methods are described in section 2.3.10, with some modifications. Seeds from each FPH

seed treatment were incubated at high temperatures (45^{o}C) for 96 hours and 100% RH. Seeds were then grown according to the warm germination protocol previously described. Percent germination was determined after 12 days.

3.3.10 Total phenolics

Total phenolic content of watermelon sprout tissue was measured using the Folin-Ciocalteau reagent method as outlined in Andarwulan and Shetty (1999) and described in section 2.3.11.

3.3.11 Statistical analysis

Statistical analysis was completed using Minitab® 16 software (Minitab ®, PA, USA). All data was subjected to analysis of variance (ANOVA) and Fisher's test to determine differences among observed means. Data sets with P-values of $P<0.05$ were deemed statistically different.

3.4 Results and Discussion

The DH values of the FPH ranged from 23.3% to 36.3%, depending on length of hydrolysis and enzyme used (Table 2). Papain produced FPH with a larger DH than either trypsin or pepsin, with a DH of 36.3% after 10 hours of hydrolysis. Trypsin yielded FPH with the next largest DH, ranging from 25.6% at 1 hour of hydrolysis to 27.5% at 10 hours of hydrolysis. Pepsin produced FPH with a larger DH at 10 hours of hydrolysis (28.4%) than trypsin, however yielded smaller DH values at 1 hour of hydrolysis (23.3%) and 5.5 hours of hydrolysis (23.5%).

The SDS-PAGE results (Figure 1) confirmed pepsin had the largest intact peptides, as the most prevalent banding formed around 200 kDa after hydrolysis at 1 hour. In contrast, papain displayed heavy banding below 36kDa for all hydrolysis times. Trypsin peptides formed bands below 200kDa.

Free amino acid composition varied depending on enzyme and hydrolysis time (Appendix A). High levels of PRO was detected in FPH-P at 5.5 hours hydrolysis (8.61g/100 g protein). VAL and GLY were also found in large quantities in the FPH treatments.

The amino acids that appeared to positively influence weight of the seedlings included TRP, MET, TYR and ASP. FPH-Pa at 10 hours of hydrolysis yielded significantly heavier seedlings (1.04 g/seedling) as compared to the water control (0.915 g/seedling) (Figure 8). FPH-T at 10 hours of hydrolysis produced significantly smaller seedlings (0.86 g/seedling) as compared to water control seedlings. Both FPH-Pa at 10 hours of hydrolysis, (which yielded significantly heavier seedlings) and FPH-T at 10 hours of hydrolysis (which yielded significantly lighter seedlings) had larger % DH values than other FPH treatments. Smaller peptides and free amino acids are more bioavailable to the plant cell and can impact cellular metabolics more effectively (Randhir, Lin, & Shetty, 2004)). Therefore, amino acid composition must play a role in the discrepancy between treatments. FPH-T at 10 hours of hydrolysis had the highest content of free VAL, GLN and LYS compared to all other treatments. FPH-Pa at 10 hours of hydrolysis had the highest content of free TRP, MET, TYR and ASP compared to all other FPH treatments. The length of the seedling appeared to be positively influenced by PRO and THR. FPH-P at 5.5 hours of hydrolysis yielded significantly longer seedlings (19.2 mm) than water

primed control seedlings (15.5 mm) (Figure 9). FPH-Pa at 1 hour of hydrolysis yielded significantly shorter seedlings (15.0 mm) than the water control.

The longest seedlings, produced by FPH-P at 5.5 hours of hydrolysis, produced a % DH of 23.5%. This was comparable to the % DH value for FPH-Pa at 1 hour of hydrolysis (23.8%), which produced the shortest seedlings. FPH-Pa treatments contain smaller peptides (below 36kDa) compared to FPH-P treatments. We would expect FPH-Pa to be more bioavailable to the plant; therefore we can infer the discrepancy in lengths is due primarily to amino acid composition. FPH-P at 5.5 hours of hydrolysis contains the highest levels of PRO, THR, HIS and GLY compared to all other FPH treatments. FPH-Pa at 1 hour of hydrolysis contains the lowest levels of VAL and GLU compared to all other FPH treatments.

Cold germination significantly affected the ability of the seedlings to germinate (Table 3). FPH-Pa at 1 hour of hydrolysis (44%), FPH-P at 5.5 hours of hydrolysis (43%) and FPH-T at 5.5 hours of hydrolysis (37%) did significantly increase the percent germination of the seedlings as compared to the water-primed seedlings (no germination). Under accelerated aging conditions, many of the FPH treatments enhanced the total percent germination of the seedlings (Table 3). FPH-Pa at 10 hours of hydrolysis (77.24%), FPH-P at 1 and 10 hours of hydrolysis (60.47% and 48% respectively), FPH-T at 5.5 and 10 hours of hydrolysis (58% and 48% respectively) all increased the percent germination of watermelon seedlings as compared to the water control seedlings (26%). High levels of the amino acids, TRP, MET, TYR, ASP, ALA, ARG, PHE, VAL, GLN, SER, GLU and LYS were all present in treatments that improved germination under stressful conditions.

Under normal germination conditions, treatments containing VAL, LYS and GLN appeared to positively effect germination. Seedlings treated with FPH-T at 10 hours hydrolysis had higher total percent germination (94%) than water controls (86%) (Table 3). FPH-P at 1 hour of hydrolysis however caused a decrease in percent germination (77%) as compared to water controls. FPH-T at 10 hours of hydrolysis contained the highest content of VAL, GLN and LYS. FPH-P at 1 hour of hydrolysis contained the highest level of ALA, SER and GLU. We can make the assumption that the discrepancy in efficacy may be not only due to the amino acid composition but the size of the peptides; FPH-T at 10 hours has smaller peptides and a greater %DH (27.5%) than FPH-P at 1 hour (23.3%), making FPH-T at 10 hours more bioavailable to the plant cells, thus stimulating growth.

Treatments that elicited multiple (2 or more) seed vigor responses contained the highest amounts of the free amino acids TRP, MET, PRO, TYR, THR, VAL, LYS, and ASP. The availability of amino acids under normal conditions is essential for plant growth, as they contribute to protein synthesis and can increase growth rate when supplemented in limited quantities (Atilio J & Causin, 1996). When supplied in conjunction with sufficient nitrogen concentrations, some exogenous free amino acids are transferred to plant sinks (ie. leafs and fruits) for increased growth (Atilio J & Causin, 1996; Fowden, 1963). FPH treatments as examined in this study are excellent sources of nitrogen and free amino acids. The discrepancy in the efficacy of these treatments on watermelon seedling vigor can be attributed to their variation in free amino acid composition generated by the enzyme and time of hydrolysis used to produce the FPH. Certain exogenous amino acids can be beneficial to plant growth(Gamborg, 1970; Rai, 2002). PRO has been found in

previous studies to stimulate the proline-linked pentose phosphate pathway (PPP), which is linked to growth (Horii, McCue, & Shetty, 2007b; Randhir, Kwon, & Shetty, 2009; Randhir, Lin, & Shetty, 2004). An increase in total phenolic content within the plant is observable when this pathway has been stimulated. In this study, no difference between treated watermelon seedlings and water control seedlings was observed, thus indicating the PPP was not stimulated. However, PRO also contributes to osmoregularity. Previous studies have demonstrated the ability of PRO to relieve salt toxicity in barley and tomatoes through promotion of K^+ uptake (Heuer, 2003; Horii, McCue, & Shetty, 2007a; Rai, 2002). Other exogenous amino acids, including ARG, ASP, VAL and GLU are able to increase K^+ and Ca^{++} uptake by 20-60%, improve osmotic stress tolerance and augment PRO functionality in osmoregularity (Atilio J & Causin, 1996; Heuer, 2003; Rai, 2002). The ability to osmoregulate in plant cells is crucial for growth in stressful environments (Shepherd & Davies, 1994). The high content of amino acids such as PRO, VAL and ASP in treatments that enhanced germination indicates osmoregularity by amino acid may play a role in mitigating stressors that could lead to deterioration during germination.

The treatment FPH-Pa at 1 hour of hydrolysis yielded significantly lower seedling lengths as compared to the water control seedlings. Though FPH-Pa at 1 hour of hydrolysis did not contain a free amino acid with a greater value than other treatments, FPH-Pa at 1 hour contained the lowest level of VAL and GLU, indicating these amino acids might be required for adequate growth. FPH-T at 10 hours of hydrolysis yielded seedlings with significantly lower weights as compared to the water control seedlings. FPH-T at 10

hours contained the highest levels of VAL, GLN and LYS, which, despite appearing to be beneficial to initial germination, may be detrimental to the production of cellular structures involved in further growth. FPH-P at 1 hour of hydrolysis negatively effected warm germination as compared to water controls. FPH-P at 1 hour contained the highest levels of ALA, SER and GLU as compared to the other treatments. Despite their positive effect on germination of heat stressed seedlings, the free amino acid composition of FPH-P at 1 hour of hydrolysis does not appear to be beneficial to warm germination of seedlings.

Amino acids can be detrimental to plant growth. The FPH treatments, FPH-T at 10 hours of hydrolysis, FPH-Pa and FPH-P at 1 hour of hydrolysis caused a decrease in vigor (ie. weight, length and warm germination). In this study, the amino acids VAL, GLU, GLN, ALA, SER, or LYS appear to be the contributing factors. GLU, ALA in particular can decrease the ability of the root to uptake NO_3^-(Muller & Touraine, 1992). LYS and THR can work cooperatively to inhibit growth (Gamborg, 1970; Miflin, 1969). SER is able to release LYS and THR through metabolic pathways thus contributing to further growth inhibition (Atilio J & Causin, 1996; Gamborg, 1970). Some treatments that elicited positive effects contained similar amounts of amino acids found to negatively impact growth. This could be attributed to the mitigation of negative amino acid effects by the presence of other amino acids. The addition of MET has been proven to mitigate the inhibitory effects of LYS and THR; the addition of ILE alleviates inhibition by VAL (Gamborg, 1970; Miflin, 1969; Muller & Touraine, 1992).

3.5 Conclusion

It appears that the presence of proline exclusively in FPH treatments may not be the determining factor in seedling growth stimulation as previously reported and observed in our soybean study. Other factors, such as size of the peptides, plant physiology, degree to which the protein was hydrolyzed and amino acid composition may play a greater role in the efficacy of FPH treatments on seedling growth. Hydrolyzed proteins are more effective at stimulating seedling growth in watermelons. Additionally, FPH treatments containing higher levels of the free amino acids TRP, MET, PRO, TYR, THR, VAL, LYS, and ASP appear to enhance growth. Further studies would determine the mechanism by which the FPH and specific amino acid combinations interact with metabolic pathways in the seedling, stimulating growth.

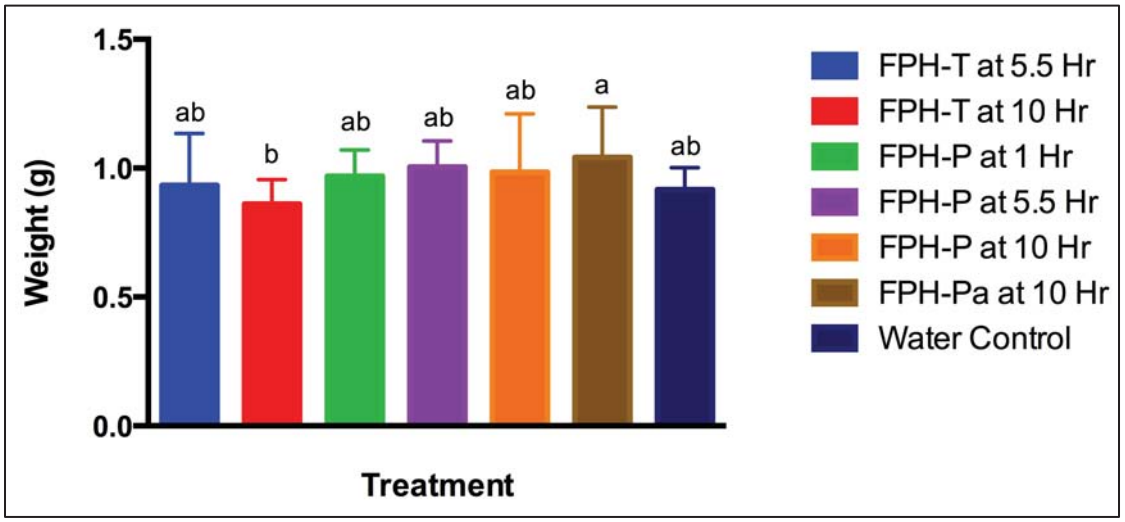

Figure 8. Effect of FPH treatments on watermelon seedling weights as compared to water control seedlings. Bars represent average values +/- SD.. Bars with different letters (a, b) indicate s significant difference ($P < 0.05$). Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

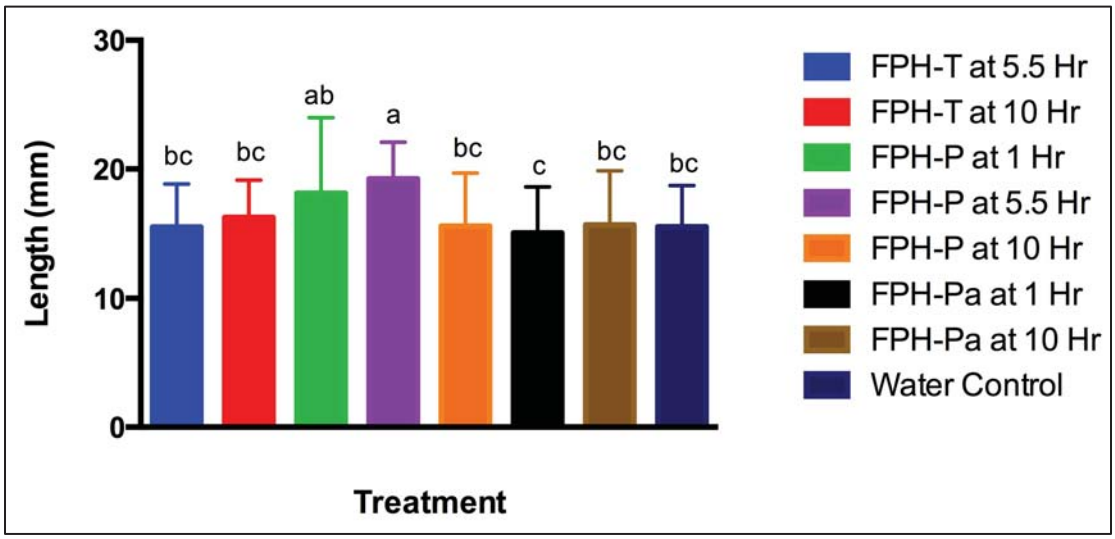

Figure 9. Effect of FPH treatments on watermelon seedling weights as compared to water control seedlings. Bars represent average values +/- SD Bars with different letters (a, b) indicate significant difference ($P < 0.05$). Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

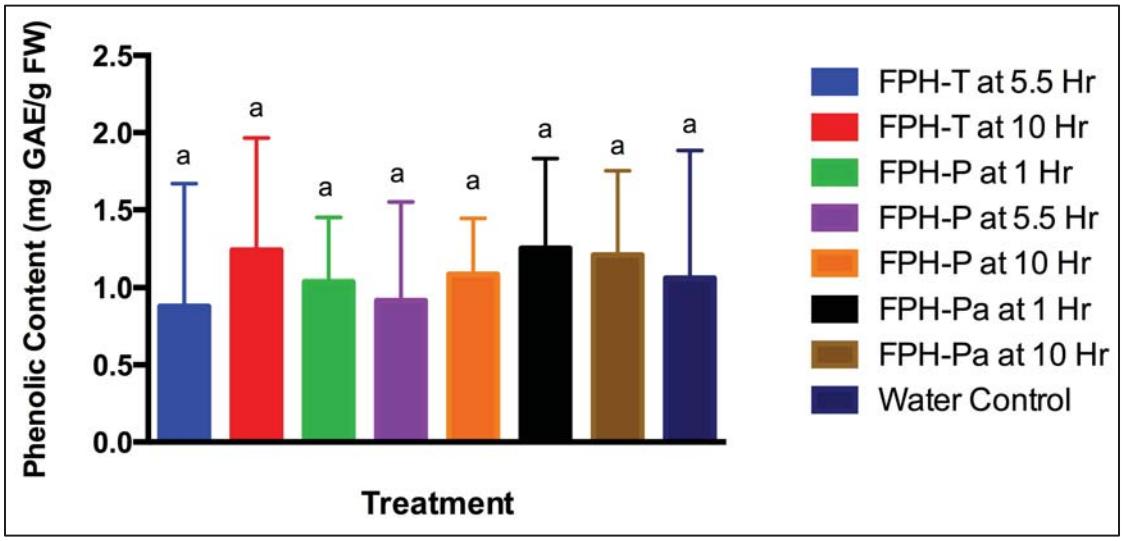

Figure 10. Effect of FPH treatments on total phenolic content in mg Gallic Acid Equivalents (GAE) of watermelon seedlings as compared to water control seedlings. Bars represent average values +/- SD. Bars with different letters (a, b) indicate significant difference ($P < 0.05$). Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

Table 3 Average percent germination[a] of seedlings by treatment

Average percent germination (%) per treatment by vigor test				
Treatment[b]	Hydrolysis Time (hours)	Warm Germination	Cold Germination	Accelerated Aging
FPH-P	1	77 (4.2)[b]	0 (0)[b]	60.57 (4.9)[ab]
	5.5	90 (5.7)[a]	44(11.3)[a]	17.71 (5.2)[c]
	10	88 (0)[a]	12 (0)[b]	57.21 (4.5)[b]
FPH-T	5.5	84 (5.66)[ab]	37 (12.7)[a]	58 (5.7)[ab]
	10	94 (2.8)[a]	0 (0)[b]	48 (8.5)[b]
FPH-Pa	1	89 (7.1)[a]	43 (15.6)[a]	N.D.
	10	89 (7.1)[a]	9 (1.4)[b]	77.23 (1.1)[a]
Water Control		86 (0)[ab]	0 (0)[b]	26 (17.0)[c]

[a] Values in parentheses represent the standard deviation associated with each data set. Data marked by different superscript letters (a,b) indicate significant differences at $P < 0.05$.

[b]FPH-P = pepsin hydrolyzed proteins, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed proteins, Water control = seeds soaked in distilled water.

CHAPTER 4. ANTIMICROBIAL EFFECT OF SILVER CARP PEPTIDES ON THE SEED BORNE PATHOGENS *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS*, *ACIDOVORAX AVENAE* SUBSP. *CITRULLI* AND *RHIZOCTONIA SOLANI*

4.1 Abstract

Antimicrobial peptides (AMPs) have been identified in many aquatic species. Peptides derived from the invasive fish specie, silver carp, were investigated for their antimicrobial efficacy against two bacterial seed borne pathogens, *Xanthomonas campestris* pv. *campestris*, and *Acidovorax avenae* subsp. *citrulli* and the fungus *Rhizoctonia solani*. Carp protein was hydrolyzed using the digestive enzymes, pepsin (FPH-P), papain (FPH-Pa) and trypsin (FPH-T) for 1, 5.5 or 10 hours to produce the peptide hydrolysates used as treatment solutions; only peptides below 3 kDa were used. Results from minimum inhibitory concentration (MIC) assay indicated the efficacy of the treatments were specific to the test organism. In contrast peptide solutions were most effective in inhibiting growth of *R. solani* at a MIC of 70 µg/mL, followed by *A. avenae* subsp. *citrulli* at a MIC of 40 µg/mL. In contrast, peptide treatments appeared to enhance the growth of *X. campestris* pv *campestris*. Peptides derived from silver carp may have potential to be used as an economical and organic treatment against some seed borne pathogens

4.2 Introduction

Seedborne plant pathogens are a major concern in agriculture around the world. Many seedborne pathogens are highly transmissible via transfer of infected seed or plant material (Agrios, 2005; Gitaitis & Walcott, 2007). Seedborne pathogens can also survive for long periods of time on their transfer medium (Gitaitis & Walcott, 2007). The bacterial plant pathogens *Xanthomonas campestris* and *Acidovorax avenae* subsp. *citrulli* along with the soilborne fungus *Rhizoctonia solani* are ubiquitous pathogenic microbes damaging crop cultivation throughout the world (Agrios, 2005; Gitaitis & Walcott, 2007; Paulitz, Smiley, & Cook, 2002).

X. campestris pv. *campestris* is a bacterial seedborne pathogen that causes the disease known as black rot of brassica crops (Radunović & Balaž, 2012; Wulff, Mguni, Mortensen, Keswani, & Hockenhull, 2002). Infected plants can be asymptomatic however, which makes control of this disease difficult and consequently spread of *X. campestris* pv. *campestris* can be extensive (Gitaitis & Walcott, 2007). Control methods for *X. campestris* pv. *campestris* are limited to cultural practices, such as use of clean seeds and *X. campestris* pv. *campestris* resistant crop varieties. New biocontrol methods such as application of antagonistic microbes are being investigated as alternative controls for *X. campestris* pv. *campestris* (Gitaitis & Walcott, 2007; Qian, Jia, Ren, He, Feng, Lu, et al., 2005; Wulff, Mguni, Mortensen, Keswani, & Hockenhull, 2002).

Acidovorax avenae subsp. *citrulli* also is a highly transmissible seedborne bacterial pathogen, which specifically affects cucurbits, particularly watermelon, causing the disease bacterial fruit blotch (BFB) (Gitaitis & Walcott, 2007; Schaad, Postnikova, & Randhawa, 2003). BFB causes water-soaked lesions on leaves and fruits, ultimately

leading to severe fruit rot (Schaad, Postnikova, & Randhawa, 2003). This pathogen has been found in several countries around the world, decimating major economic melon crops (Schaad, Postnikova, & Randhawa, 2003). Current control of BFB involves the use of clean seed, proper storage of seeds, and extensive testing of seeds prior to planting. Testing involves a polymerase chain reaction assay (PCR) of seed washings and/or a grow out no less than 10,000 seeds with a zero-tolerance disease incident (Gitaitis & Walcott, 2007; Society, 2014).

R. solani is one of the most common, broad host soilborne plant pathogens (Sneh, 1996). It affects numerous crops causing root, collar and stem rots of soybeans, wheat, canola, tomatoes and numerous other species. It can also cause foliar blights in crops such as cabbage and turfgrass (Blazier & Conway, 2005; Sneh, 1996). Methods to control *R. solani* involve chemical application of fungicides or fumigants to the soil or seed prior to planting (Blazier & Conway, 2005; Dhingra, Costa, Silva Jr, & Mizubuti, 2004; Mancini & Romanazzi, 2013); however, the effectiveness of these treatments is limited because of the resistant, long-lived dormant resting structures (sclerotia) produced by *R. solani*. Preventative methods, such as sanitation of farm equipment, use of new seed (ie. not saving or using seeds from previous years' crops), exclusion of the pathogen (often involving quarantine of infected materials and using certified clean seed are essential in control of seed borne pathogens (Gitaitis & Walcott, 2007; Mancini & Romanazzi, 2013; Maude, 1996; Society, 2014). Seed treatments, such as salt-water brines, hot water and chemical treatments are also used for disinfection and preventing infection (Agrios, 2005; Maude, 1996). Current methods of prevention and disinfesting/disinfecting are not always effective however (Gitaitis & Walcott, 2007). Chemical methods of seed

treatment are often expensive, can affect the ecology of the environment surrounding the seed and are not successful against some bacterial pathogens (Agrios, 2005). This is particularly true for certified organic crop producers who have limited options for seed treatments. Copper dusts and hot water baths are the most common organic methods for controlling seed borne, however both methods are less effective than chemical methods and under some conditions can be phytocidal (Agrios, 2005).

Peptides isolated from plant, animal, or microbial sources have been shown to inhibit the growth of various bacteria, fungi (Di Bernardini, et al., 2011; Hancock & Chapple, 1999). Antimicrobial peptides (AMPs) are classified into 5 groups based on their structure and composition; α-helical AMPs, β-sheet AMPs, cysteine rich AMPs, AMPs rich in regular amino acids and AMPs rich in rare, modified, amino acids (Brogden, 2005; De Lucca & Walsh, 1999; Radunović & Balaž, 2012). Though each group has a unique mode of action against microbial cells, they all share some similarities. AMPs are typically less than 3 kDa in size and contain between 15 and 38 amino acid residues (Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). Cationic and amphipathic or hydrophobic peptides are the most commonly used AMPs for microbial inhibition and they are involved anionic envelope or phospholipid membrane disruption (Ali & Reddy, 2000; Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). AMPs may be specific to individual plant, fungal or bacterial species or be effective against a broader species range (Ali & Reddy, 2000).

AMPs have been isolated from many aquatic organisms (Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010), but little work has been done with fresh water fish species. Silver carp or ‘Asian carp’ (*Hypophthalmichthys molitrix)* (Kolar, Chapman,

Courtenay Jr, Housel, Williams, & Jennings, 2005) is an invasive fish species residing in the Wabash River in Indiana and comprises over 60 percent of the total fish biomass of the Illinois River (ACRCC, 2012; Minnesota DNR, 2005). Concerns have been raised over the destruction of river ecology due to the rapidly expanding Asian carp population and the aggressive nature of the fish (Minnesota DNR, 2005; Roth & Secchi, 2012). Control of the fish is currently limited to their harvest, as other picicidal options, such as electro-chemical approaches, are ineffective (Roth & Secchi, 2012). Poor consumer acceptance and low market values are attributed to their boney composition which prevents Asian carp from being accepted as a food source (Freeman, 1999; Roth & Secchi, 2012).

The proposed use of AMPs from silver carp as a seed treatment against seedborne pathogens is investigated in this study. Previous studies have indicated protein hydrolysates derived from fish (FPH), have stimulatory effects on the growth of some plants when applied to seeds (Andarwulan & Shetty, 1999). This is important as it suggests that there is a low likelihood that will be phytotoxic when applied to seeds. Two common seedborne bacterial pathogens, *Xanthomonas campestris* pv. *campestris*, *Acidovorax avenae* subsp. *citrulli* and the soilborne fungal plant pathogen *Rhizoctonia solani*, were used for this investigation.

4.3 Materials and Methods

4.3.1 FPH preparation

Silver carp meat was hydrolyzed using the enzymes papain, pepsin and trypsin and hydrolyzed for 1, 5.5 or 10 hours. The treatments, papain hydrolyzed protein (FPH-Pa),

pepsin hydrolyzed protein (FPH-P) and trypsin hydrolyzed protein (FPH-T) were prepared according to Liceaga-Gesualdo & Li-Chan (1999) with modifications as described in section 2.3.1. The FPH treatments best able to elicit vigor responses from preliminary seed vigor trials (data not shown) were chosen for further testing.

4.3.2 Degree of hydrolysis (DH)

The DH of the FPH samples was determined based on the trinitrobenzene sulfonic acid (TNBS) method as described by Adler-Nissen (1986) with modifications as described in section 2.3.2.

4.3.3 Free amino acid composition

Free amino acid composition analysis of FPH samples was performed by the Bindley Discovery Park Proteomics Facility at Purdue University using a Beckman HPLC (126 pump, 166 detector, 507 autosampler) with a Waters AccQ Tag amino acid analysis column and UV detector, calibrated with amino acid standards (Pierce, Rockford, IL).

4.3.4 FPH solution preparation

For the agar diffusion assay, FPH were sterilized by filtration using a 0.22 μm filter and stored at -80^{o}C for further use. For the minimum inhibitory concentration assay, FPH were separated by size fractions into peptides larger than 3 kDa and peptides equal or less than 3 kDa using Amicon Ultra-15 centrifugal filter units (EMD Millipore

Corporation, MA, USA). FPH were centrifuged at 4000xg and 25°C for 40 min. Peptide fractions equal to or below 3 kDa were collected and used immediately. Solutions were prepared in concentrations of 40, 70 and 100 µg protein in sterile distilled deionized water (w/v). Each solution was filter sterilized twice using a 0.20 µm filter and stored at -20°C for further use.

4.3.5 Culture preparation

Acidovorax avenae subsp. *citrulli* was isolated from diseased watermelon and provided by Dr. Dan Egel at the Southwest Purdue Agricultural Center (Vincennes, IN, USA). Cultures of *A. avenae* subsp. *citrulli* were transferred into lysogeny broth (LB) (Thermo Fisher Scientific Inc, Waltham, MA) and incubated for 24 hours at 25°C for use in the minimum inhibitory concentration (MIC) assay and the agar diffusion assay. *Xanthomonas campestris* pv. *campestris* was isolated from cabbage and provided by Dr. Lori Hoagland, Purdue University (West Lafayette, IN, USA). *X. campestris* pv. *campestris* was transferred into a yeast dextrose broth and incubated for 24 hours at 25°C. Dr. Lori Hoagland also provided *Rhizoctonia solani*, isolated from turfgrass seed (West Lafayette, IN, USA). *R. solani* cultures were transferred into a potato dextrose broth, mixed thoroughly with the liquid medium, and incubated for 24 hours at 25°C. *R. solani* and *X. campestris* pv. *campestris* cultures were used for the MIC assay.

4.3.6 Agar disk-diffusion assay

Sterile chromatography paper disks (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) 6 mm in diameter were impregnated with sterile FPH solutions.

Solutions contained 20 mg protein/mL sterile distilled deionized water. A disk impregnated with tetracycline (3 mg tetracycline/mL sterile distilled deionized water) was used as a positive control. Likewise, a disk without any treatment was used as a negative control. Disks were dried in Petri dishes for 3 hours before use. *A. avenae* subsp. *citrulli*, adjusted to an optical density (O.D.) of 0.5 at 600 nm, was spread on LB agar and the treated disks were placed on agar and incubated for 24 hours at 37^{o}C.

4.3.7 Minimum inhibitory concentration assay (MIC)

The MIC assays were performed based on the protocol outlined by Liceaga-Gesualdo, Li-Chan, & Skura (2001) with some modifications. MIC assays were conducted in sterile 96-well plates (Nunc™ plates, Thermo Fisher Scientific Inc, Waltham, MA). To each well was added 100 µL of broth and the respective cultures, FPH treatments in concentrations of 40, 70 or 100 µg/mL and 25 µL of *X. campestris* pv. *campestris*, *A. avenae* subsp. *citrulli* or *R. solani*. Cultures were diluted to an O.D. of 0.5 before well plate inoculation. Sterile distilled deionized water was added, bringing the total volume to 250 µL per well for all treatments. Controls included a sterile broth (containing only nutrient media), a sterile treatment control (containing broth, FPH and sterile distilled deionized water without inoculation of a culture) and a negative culture control (containing broth, sterile distilled deionized water and inoculant without FPH treatment). Six replicates for each treatment and controls were used. Plates were covered with a sterile 96-well-plate lid and incubated at 35^{o}C The O.D. at 600nm was measured using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific Inc., Waltham, MA) every 12 hours for 60 hours.

4.3.8 Statistical Analysis

Data for the MIC assay was analyzed using a one-way ANOVA followed by Fisher's multiple comparisons test using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Statistical significance was established at $\alpha = 0.05$ unless otherwise indicated.

4.4 Results and Discussion

4.4.1 Peptide characterization

Hydrolysis of the fish protein yielded peptides that varied from 23.3% DH to 36.3%, depending on enzyme and length of hydrolysis. Pepsin yielded peptides with a lower DH and higher overall molecular weights as confirmed by SDS-PAGE, with bands forming around 200 kDa after hydrolysis at 1 hour (Figure 1). The most prevalent banding occurred around 36kDa for FPH-P at 10 hours of hydrolysis. Trypsin hydrolyzed proteins produced slightly smaller peptides (banding observed at less than 200 kDa) but with greater DH values than pepsin-produced peptides. Papain hydrolyzed the protein more extensively than both pepsin and trypsin. Papain produced proteins with the highest DH value (36.28% after 10 hours of hydrolysis) and the lowest molecular weight peptides, with dense banding occurring below 36kDa for all hydrolysis times. Free amino acid composition was determined for all treatments (Appendix A).

4.4.2 Agar disk diffusion

The agar disk diffusion assay yielded no growth inhibition of *A. avenae* subsp. *citrulli*. A zone of inhibition was observed for the tetracycline control, however no zone of inhibition was visible for any FPH treatment (Figure 11). The fraction of peptides with the greatest antimicrobial activity typically are those below 3 kDa in weight (Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). In our agar disk diffusion assay, the lack of microbial inhibition was likely due to the dilution of peptides below the 3 kDa by the higher mass fractions and thus were ineffective against the *A. avenae* subsp. *citrulli*.

4.4.3 MIC assay: *Rhizoctonia solani*

Peptides below 3 kDa were used to produce the treatments for MIC trials. Treatments varied in their efficacy depending on incubation time. Some FPH preparations were only effective until 24 hours as in the case of FPH-C (6.43% growth inhibition). Others, such as FPH-T at 1 hour and 5.5 hours of hydrolysis were only effective at 48 hours at 70 μg/mL (7.21% growth inhibition) and 70 and 100 μg/mL respectively (5.96% and 6.91% inhibition). The most consistent treatments, which suppressed microbial growth over the entire incubation period of 72 hours, were FPH-P at 1 hour of hydrolysis (100 μg/mL) and 5.5 hours of hydrolysis (70 μg/mL until 48 hours; 100 μg/mL for entire incubation time) (Figure 12). Inhibition of growth compared to the control was greatest at 24 hours for both treatments, with 17.9% growth inhibition for FPH-P at 1 hour of hydrolysis (100 μg/mL) and 18.6% inhibition (100 μg/mL) for FPH-P at 5.5 hours of hydrolysis. No treatments were able to inhibit *R. solani* growth at

concentrations below 70 µg/mL. Antimicrobial peptide (AMP) fractions that appear effective against *R. solani* were those below 3 kDa in weight and released by pepsin after 1 hour (DH of 23.3%) or before 5.5 hours (DH of 23.5%) of hydrolysis.

A common mechanism for fungal inhibition by AMPs involves creation of a pore in the fungal cell membrane (Brogden, 2005). This requires the AMP involved to traverse the membrane and larger peptides are more effective (Brogden, 2005). Growth inhibition of *R. solani* by FPH-P at 1 hour and 5.5 hours of hydrolysis could be attributed to the relatively low DH and larger proportion of intact peptides as compared to other treatments. Another method by which the peptides could be operating against the *R. solani* involves the 'carpet like mechanism' theory in which peptides lie parallel to the surface of the phospholipid bilayer of the microbial membrane (Ali & Reddy, 2000; López-García, González-Candelas, Pérez-Payá, & Marcos, 2000). Pore formation in the membrane, and thus cell lysis and death, is only achieved when a threshold concentration of amphiphillic AMPs is reached (Ali & Reddy, 2000; López-García, González-Candelas, Pérez-Payá, & Marcos, 2000). The higher concentrations (70 and 100 µg/mL) required to achieve antifungal effects, as observed in this study, may be due to this 'carpet-like mechanism'. Alternatively, higher antifungal effects in peptides have been attributed to aromatic and cysteine-rich peptides (Liu, Dong, Xu, Zeng, Song, & Zhao, 2008; Liu, Zeng, Dong, Xu, Song, & Zhao, 2007). Pepsin, as used in FPH-P production, has a broad specificity and will preferentially cleave aromatic residues (Worthington & Worthington, 2011). We can also attribute some inhibition of *R. solani* to aromatic residues present in FPH-P treatments. Both FPH-P at 1 hour and 5.5 hours of hydrolysis contained aromatic free amino acids. FPH-P at 1 hour of hydrolysis contained phenylalanine (PHE)

(2.12mg/100 mg protein), tryptophan (TRP) (0.77mg/100 mg protein), and tyrosine (TYR) (1.36mg/100 mg protein). FPH-P at 5.5 hours of hydrolysis also contained PHE (1.78mg/100 mg protein), TRP (0.65mg/100 mg protein), and TYR (1.31mg/100 mg protein).

Interference by peptides with Ca^{2+} signaling, required for development of normal fungal hyphae growth, has been observed in previous studies (Ali & Reddy, 2000). In - our study, no examination of hyphal morphology was conducted; however, negligible antifungal activity in some treatments may have been the result of abnormal fungal growth and should be further explored.

4.4.4 MIC assay: *Acidovorax avenae* subsp. *citrulli*

Concentrations that were most effective against *A. avenae* subsp. *citrulli* varied by treatment and incubation time. The lowest concentration tested (40 μg/mL) was effective at times 36, 48 and 60 hours for both FPH-Pa 5.5 and 10 hours of hydrolysis. Concentrations of 70 μg/mL and 100 μg/mL were effective for both treatments at 36 and 48 hours, indicating that the inhibition of *A. avenae* subsp. *citrulli* growth by the FPH treatments is not concentration dependent. Preliminary studies, however, demonstrated lack of inhibition below a concentration of 40 μg/mL (data not shown). FPH treatments most effective at inhibiting *A. avenae* subsp. *citrulli* included FPH-Pa at 5.5 for incubation times of 24-60 hours, and FPH-Pa at 10 hours of hydrolysis for incubation times of 24-60 hours (Figure 13). FPH-P at 1 hour of hydrolysis was able to inhibit growth at 24 (7.87% inhibition using 70 μg/mL) and 36 hours of incubation (9.90% inhibition with 70 μg/mL). FPH-P at 5.5 hours of hydrolysis also was effective at

inhibiting growth at 36 hours incubation time (7.93% at 70 μg/mL). The greatest percent growth inhibition for FPH-Pa at 5.5 hours of hydrolysis was observed at 48 hours with 40 μg/mL (12.3% growth inhibition compared to controls). Papain hydrolysis between 5.5 hours of hydrolysis (DH of 26.3%) and 10 hours (DH of 36.3%) yielded AMPs smaller than 3 kDa in weight and had inhibitory effects against *A. avenae* subsp. *citrulli*. Smaller peptides (treatments with greater DH), such as those produced with the enzyme papain, appeared to be more effective against *A.avenae* subsp. *citrulli*. Papain has limited substrate specificity and thus produces a wide array of free amino residues and peptides (Worthington & Worthington, 2011). Destabilization of the bacterial membrane can still be achieved with smaller peptides that are cationic and amphiphillic or hydrophobic in nature (Brogden, 2005; Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). FPH-Pa at 5.5 hours of hydrolysis contains a number of free cationic amino acids, including histidine (HIS) (1.72 mg/100 mg protein), lysine (LYS) (4.66 mg/100 mg protein) and arginine (ARG) (2.85 mg/100 mg protein). This treatment also contains hydrophobic amino acids, including valine (VAL) (20.4 mg/100 mg protein), methionine (MET) (17.5 mg/100 mg protein) and proline (PRO) (3.65 mg/100 mg protein). FPH-Pa at 10 hours of hydrolysis contained the same cationic and hydrophobic free amino acids as FPH-Pa at 5.5 hours of hydrolysis. Amino acids HIS (1.42 mg/100 mg protein), LYS (4.27 mg/100 mg protein) and ARG (3.50 mg/100 mg protein), and VAL (22.2 mg/100 mg protein), MET (18.9 mg/100 mg protein) and PRO (3.91 mg/100 mg protein) were present in FPH-Pa at 10 hours of hydrolysis.

In addition to AMPs causing membrane destruction, ‘intracellular killing’ has been proposed as an alternative method of microbial inhibition by AMPs (Brogden, 2005;

Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). Intracellular killing involves peptides that are translocated synergistically with other membrane proteins or AMPs and operate within the cytoplasm of the cell. Once in the cytoplasm, the peptides can inhibit enzyme activity, nucleic acid and protein synthesis and bind DNA and RNA (Brogden, 2005; Ravichandran, Kumaravel, Rameshkumar, & AjithKumar, 2010). The assortment of peptides and amino residues produced by papain may work synergistically with integral membrane proteins, promoting intracellular killing and membrane disruption leading to the inhibition of *A. avenae* subsp. *citrulli*.

4.4.5 MIC assay: *Xanthomonas campestris* pv. *campestris*

FPH treatments were not consistently effective at inhibiting growth of *X. campestris* pv. *campestris*. Pepsin hydrolysis (FPH-P) at 1 hour of hydrolysis was effective at inhibiting growth at 12 (70 μg/mL), 24 (70 and 100 μg/mL) and 60 hours (70 and 100 μg/mL). FPH-Pa (Papain) at 1 hour hydrolysis had a similar effect and was inhibitory at times 12 hours (70 μg/mL), 24 hours (70 and 100 μg/mL) and 36 hours (100 μg/mL). FPH-P at 5.5 hours of hydrolysis inhibited growth at 24 and 36 hours at a concentration of 100 μg/mL whereas FPH-Pa at 5.5 hours of hydrolysis inhibited growth at 12 and 24 hours at a concentration of 70 μg/mL; however inhibition of growth was minimal; no more than 7.0% growth reduction was observed. In contrast, many of the FPH treatments, a significant increase in bacterial growth was observed (Figure 14). Significantly higher growth of *X. campestris* pv. *campestris* was observed with treatment FPH-Pa at 10 hours of hydrolysis at time 36 and 48 hours (100 μg/mL). Both FPH-T at 1 hour and 5.5 hours of hydrolysis increased growth from 24 to 48 hours (100 μg/mL).

FPH-T at 1 hour of hydrolysis increased growth 19.6%. Treatments FPH-T at 10 hours of hydrolysis and FPH-C increased growth of *X. campestris* pv *campestris* over the entire incubation period compared to the negative culture control. At time 60, FPH-C (100 µg/mL) increased growth 5.9%. FPH-T at 1 hour of hydrolysis (100 µg/mL) increased growth 10.2%.

Despite some growth by FPH-T and FPH-C treatments, it appears that pepsin and papain hydrolyzed proteins may yield peptides with some antimicrobial effect against *X. campestris pv. campestris*. As in the case with *R. solani*, the larger peptides produced by shorter hydrolysis times are able to span the width of a bacterial cell membrane (Brogden, 2005; Hancock & Chapple, 1999; Song, Wei, Luo, & Wang, 2012) causing pores and allowing for cell lysis, membrane disruption and intracellular killing by smaller peptides introduced to the cytoplasm (Brogden, 2005).

FPH treatments also increased the growth of *X. campestris* pv. *Campestris,* however. *X. campestris* pv. *campestris* secretes an exopolysaccharide (EPS) during growth which may have increased the absorbance of our samples (Souw & Demain, 1979; B.-Y. Yang & Tseng, 1988). Additionally, the production of the EPS by *X. campestris* pv. *campestris* can be enhanced by growth factors, such as carbohydrates, nitrogen and amino acids (Jackson, Frymier, Wilkinson, Zorner, & Evans, 1998; Souw & Demain, 1979). Amino acids such as glutamic acid, were found to strongly stimulate EPS production (Jackson, Frymier, Wilkinson, Zorner & Evans, 1998). All FPH treatments contained between 0.98 and 4.55 mg/100 mg protein of glutamic acid. Likewise, nitrogen sources also increase EPS production and increase growth of *X. campestris* pv. *campestris* (Jackson, Frymier, Wilkinson, Zorner, & Evans, 1998; Souw & Demain,

1979). The FPH treatments appear to have stimulated growth of *X. campestris* pv. *campestris*, acting as a source of both nitrogen and free amino acids. In addition to production of an EPS, exo-proteases are secreted by *X. campestris* pv. *campestris* (Dow, Clarke, Milligan, Tang, & Daniels, 1990; B.-Y. Yang & Tseng, 1988). FPH treatments with no inhibitory or stimulatory effects may have been inactivated by exo-proteases produced by *X. campestris* pv. *campestris*.

4.4.6 Conclusion

The alternative use of the deleterious fish species, silver carp as a source of antimicrobial peptides was investigated. The efficacy of the peptides (< 3 kDa) appeared to be microorganism specific, with the best results obtained for the fungus, *R. solani* followed by *A.avenae* subsp. *citrulli.* Peptides isolated from silver carp did not appear to be effective in inhibiting the growth of *X. campestris* pv. *campestris.* For all cultures, inhibition was not substantial, thus higher concentrations of the FPH or further fractionation, purification and characterization by other methods may be required for greater growth inhibition. Additional filtration and further isolation of specific peptides involved in antimicrobial activities could benefit the efficacy of these treatments. Using peptides derived from silver carp is a potential method of controlling certain seed borne pathogens and may aid in the control of an invasive, injurious fish species.

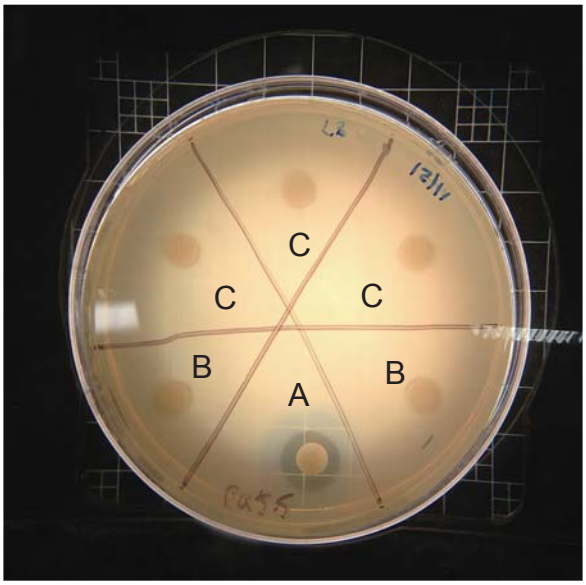

Figure 11. Plate for agar disk diffusion assay. *A.avenae* subsp. *citrulli* was grown on LB agar. Disks include tetracycline control (A), blank disks without any treatment (B) and treatment disks containing FPH-Pa at 5.5 hours of hydrolysis (C).

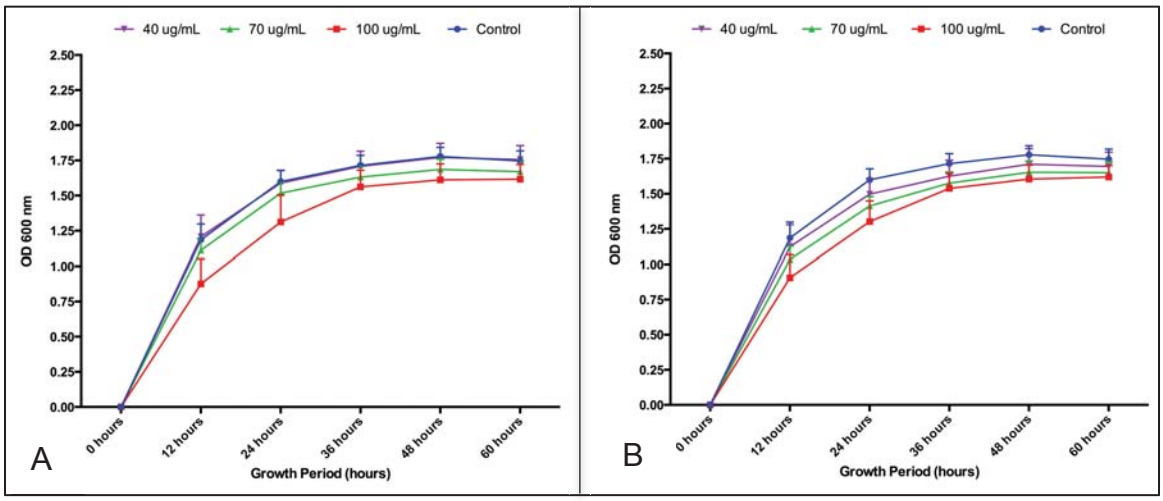

Figure 12. Effects of FPH hydrolyzed with pepsin (FPH-P) at (A) 1 hr and (B) 5.5 hrs of hydrolysis on the fungus *R. solani* at initial optical density (O.D.) of 0.5 at 600 nm. Growth measured as change in O.D.

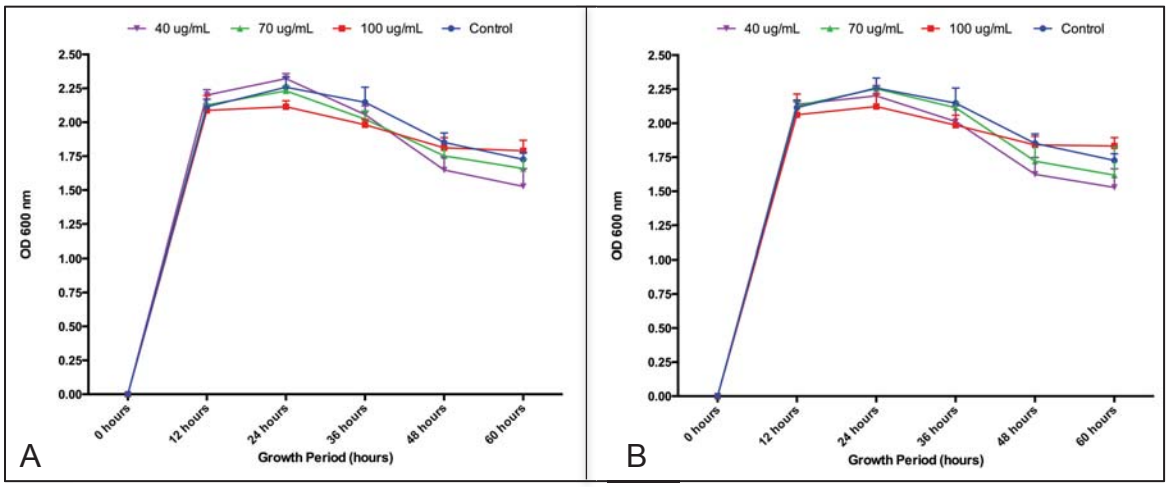

Figure 13. Effects of FPH hydrolyzed with papain (FPH-Pa) at (A) 5.5 hrs and (B) 10 hrs of hydrolysis on the growth of *A. avenae* subsp. *citrulli* at initial optical density (O.D.) of 0.5 at 600 nm. Growth measured as change in O.D.

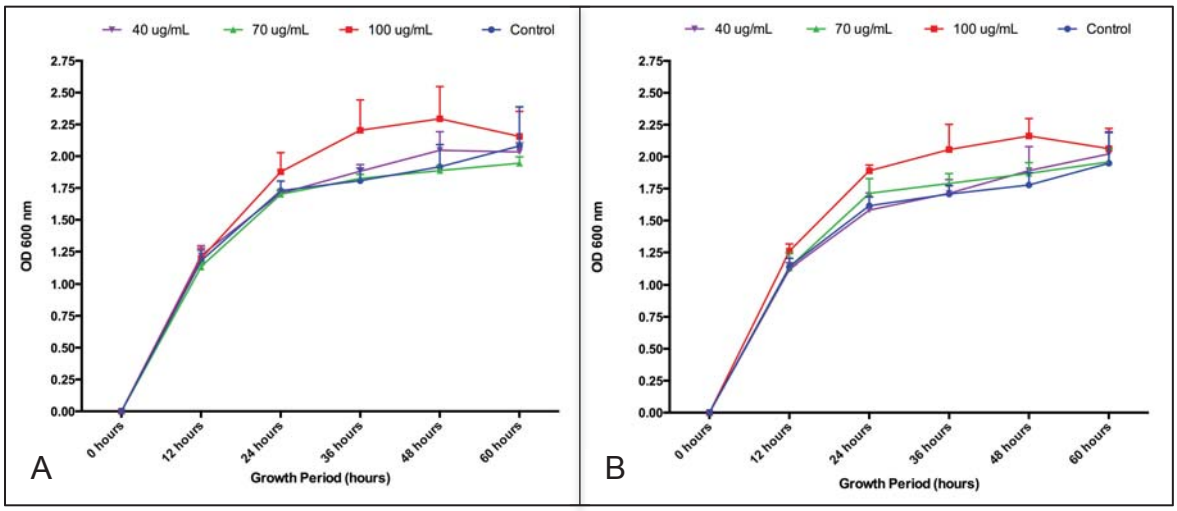

Figure 14. Effects of FPH hydrolyzed with trypsin (FPH-T) at (A) 1 hr of hydrolysis and (B) unhydrolyzed protein (FPH-C) on the growth of *X. campestris* pv. *campestris* at initial optical density (O.D.) of 0.5 at 600 nm. Growth measured as change in O.D.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusion

Invasive silver carp populations are spreading in the Mississippi River System at a rapid rate. Methods to control and eradicate this species are limited to chemical and electrochemical approaches, which have been so far ineffective. Use for the fish in culinary applications has also been limited in success due to their poor consumer acceptability. Other uses for the fish, and more specifically the protein, have been investigated for their efficacy as cryoprotectants and bioactive components in food systems. Additionally, silver carp proteins may be effective as a seed treatment by enhancing seed vigor and acting against seed borne diseases.

In this project, FPH were prepared using silver carp meat and the enzymes trypsin, pepsin and papain in a 2.5% enzyme/substrate (protein) ratio. The protein was hydrolyzed for 1, 5.5 or 10 hours. In the first study, the FPH were investigated for their stimulatory effects on soybean seedling vigor. Tests for vigor included seedling weight, length, total phenolic content, guiaicol peroxidase content, warm germination, cold germination and accelerated aging. The hydrolysates, FPH-P and FPH-Pa at 1 hour of hydrolysis elicited greater vigor responses than did the water primed seedlings. More specifically, higher total phenolic and guiaicol peroxidase content, and higher percent

germination (in both warm and accelerated aging germination) were observed in seedlings primed with FPH-P and FPH-Pa at 1 hour. It appears that the presence of proline and synergy between other amino acids such as glutamic acid found in the FPH elicits the proline-linked pentose phosphate pathway (PPP), stimulating phenolic production and growth.

In the second study, stimulation of watermelon seedling vigor when FPH was applied as a seed dressing was investigated. Similar tests were used to evaluate vigor, including seedling weight, length, total phenolic content, warm and cold germination and accelerated aging. No stimulation of the PPP was observed, as phenolics did not differ between water-primed seedlings (control) and FPH treated seedlings, however all other aspects of watermelon vigor was increased by certain FPH treatments. Variations in free amino acid composition, and degree to which the protein has been hydrolyzed (%DH) may be determining factors in the efficacy of FPH treatments on vigor augmentation of watermelon seeds. Specifically, FPH treatments with larger %DH and a higher content the amino acids TRP, MET, PRO, TYR, THR, VAL, LYS and ASP appear to be beneficial in mitigating environmental stressors and stimulating growth. PRO, which had previously been thought to be an essential amino acid in vigor stimulation in seedlings, may not be imperative in a watermelon seedling system.

In the final study, the antimicrobial efficacy of the peptides was investigated against three prevalent seed borne pathogens; *X. campestris* pv. *campestris*, *R. solani*, and *A. avenae* subsp. *citrulli*. A minimum inhibitory concentration method was used to determine percent decrease in growth as compared to bacterium growth without FPH treatment. FPH treatments FPH-C and FPH-T at 10 hours of hydrolysis increased the growth of *X. campestris pv. campestris*. Amino acids can act as growth factors and aid in the production of EPS. The growth of *R. solani* was decreased by FPH-P at 1 and 5.5 hours of hydrolysis. The decrease in

growth can be attributed to the degradation of membrane walls by larger, aromatic and cationic peptides. *A. avenae* subsp. *citrulli* was inhibited FPH-Pa at 5.5 and 10 hours of hydrolysis. Similarly, small, cationic peptides are involved in pore formation in bacterial cell envelopes. A large threshold (concentration) of peptides must be reached in order for cell lysis to occur; this is called the 'carpet-like mechanism'.

5.2 Future Directions

5.2.1 Seedling Vigor Studies: Soybean and Watermelon

Investigations in the seedling vigor studies confirmed the ability of FPH treatments to increase seedling vigor and stimulate growth. Biochemical assays involving the metabolic system of the seedlings, such as glucose-6-phosphate dehydrogenase (G6PDH) quantification could provide insight as to the mechanisms of action by which the FPH operate. Further peptide characterization such as use of a high performance liquid chromatograph (HPLC) would not only assist in this endeavor, but could be beneficial in determining the FPH treatment conditions best suited for this application. Finally, the practicality of the FPH treatments should be investigated further. Shelf life of the product and stability of the FPH on the seed during storage and in the ground over longer periods of time would indicate whether this treatment could be used in a realistic situation.

5.2.2 FPH as Antimicrobials

Some of the FPH treatments inhibited growth of certain seed borne fungi and bacteria, however most cultures were not inhibited substantially. Investigation into whether the FPH at higher concentrations impede growth more significantly would determine whether the

treatments could be used in a practical application. As it appears the FPH treatments are species specific, sensitivity of other bacteria and fungi to the FPH treatments should be examined. Advanced microscopy techniques such as scanning electron microscopy (SEM) could assist in the determination of the mechanism of antimicrobial action by the FPH; HPLC techniques to further characterize the peptides would also enhance our understanding. Finally, in vivo testing (ie. application of the FPH treatments to a seed) could be completed to further determine the practicality of this application.

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APPENDICES

Appendix A

Table A. 1. Free amino acid composition of FPH treatments[a] (g/100g protein)

	FPH-T			FPH-P			FPH-Pa			FPH-C
Hours of Hydrolysis	**1[b]**	**5.5**	**10**	**1**	**5.5**	**10**	**1**	**5.5**	**10**	
Amino Acid										
PHE	3.79	6.02	3.04	2.12	1.78	3.33	3.64	5.85	3.95	0.42
ILE/LEU	5.32	6.69	5.71	4.00	3.14	4.61	6.62	8.84	5.36	0.98
TRP	1.38	1.81	1.19	0.77	0.65	1.32	1.37	1.59	1.82	0.41
MET	9.29	13.9	9.90	7.90	5.58	9.04	10.7	17.5	18.9	2.67
VAL	27.7	25.0	28.2	18.2	17.9	27.9	16.6	20.4	22.2	15.1
PRO	3.17	1.78	1.60	7.08	8.61	3.20	5.55	3.65	3.91	8.24
TYR	2.67	3.23	2.38	1.36	1.31	2.81	2.02	2.76	3.62	1.44
THR	2.07	1.81	2.49	4.01	4.08	2.18	3.51	2.46	2.50	3.05
ALA	4.17	3.22	5.16	7.20	6.17	4.10	6.56	4.75	5.30	6.64
ARG	4.06	3.94	0.11	0.98	1.03	4.19	0.32	2.85	3.50	0.26
HIS	3.94	1.91	1.88	2.77	5.11	3.75	4.59	1.72	1.42	8.56
GLN	10.5	12.9	15.05	3.92	3.21	10.5	5.20	6.20	6.62	1.78
GLY	9.72	4.60	8.49	25.2	33.3	10.7	24.4	9.43	8.90	45.4
ASN	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SER	0.80	0.73	0.71	2.21	0.39	0.78	1.38	1.18	0.99	1.10
LYS	8.46	8.00	10.37	4.89	4.12	8.93	4.83	4.66	4.27	2.78
CYS-CYS[c]	0.13	0.08	0.04	0.04	0.03	0.07	0.03	0.06	0.06	0.01
GLU	2.00	2.61	2.83	4.55	3.03	1.84	1.22	3.26	3.28	0.98
ASP	0.81	1.75	0.82	2.74	0.47	0.81	1.44	2.84	3.37	0.21

[a]FPH-P = pepsin hydrolyzed proteins, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed proteins.
[b]Hours of hydrolysis (1, 5, or 10 hrs).
[c]Cysteine is underestimated since pre-derivatization was not performed.

Appendix B: Percent growth of microorganisms growth in FPH treatments compared to control

Table B. 1. Percent growth[1] at incubation time 12 hours: *Rhizoctonia solani*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-26.2	-23.7	-10.5	-11.3	0.40	2.01	0.36	-0.30	-10.8	-16.1
70	-6.27	-12.8	-5.27	-9.65	10.1	1.12	-3.70	-1.08	-2.03	-11.9
40	1.84	-5.31	-6.05	-7.62	3.23	-8.23	-4.45	-1.00	1.22	-5.69

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 2. Percent growth[1] at incubation time 24 hours: *Rhizoctonia solani*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-18.0	-18.6	-5.41	-7.70	6.92	19.8	-1.88	-1.68	-5.23	-8.87
70	-5.12	-11.7	-1.66	-5.69	18.5	31.0	0.28	-0.68	1.10	-9.07
40	-0.67	-6.38	-3.00	-4.78	28.8	40.2	0.96	0.48	1.60	-6.43

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 3. Percent growth[1] at incubation time 36 hours: *Rhizoctonia solani*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-9.34	-9.74	-11.8	-12.11	-2.39	-3.08	-5.18	-5.96	-0.52	-0.25
70	-5.17	-7.01	-6.47	-8.60	-1.84	-0.74	-7.21	-6.91	-1.56	0.67
40	-0.36	-3.77	-0.69	-5.16	1.52	0.95	-1.82	-5.03	1.63	1.71

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 4. Percent growth[1] at incubation time 48 hours: *Rhizoctonia solani*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-7.45	-7.25	-2.01	-3.19	-5.72	-6.22	-0.95	-1.95	-4.29	-4.67
70	-4.37	-5.52	-0.81	-1.64	-5.24	-6.29	-1.84	-1.13	-4.81	-4.69
40	0.55	-2.99	1.89	0.55	-0.15	-3.27	1.06	0.92	-0.60	-3.23

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 5. Percent growth[1] at incubation time 60 hours: *Rhizoctonia solani*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-3.50	2.35	-5.26	5.59	-1.29	-2.51	-9.12	1.05	-2.55	N.D.
70	-6.42	3.96	-2.38	-10.46	0.61	0.54	-2.17	-0.33	-2.16	N.D.
40	8.35	14.4	2.12	6.05	4.06	1.09	0.66	-36.7	0.59	N.D.

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 6. Percent growth[1] at incubation time 12 hours: *Acidovorax avenae subsp. citrulli*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-3.50	2.35	-5.26	5.59	-1.29	-2.51	-9.12	1.05	-2.55	N.D.
70	-6.42	3.96	-2.38	-10.46	0.61	0.54	-2.17	-0.33	-2.16	N.D.
40	8.35	14.4	2.12	6.05	4.06	1.09	0.66	-36.7	0.59	N.D.

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 7. Percent growth[1] at incubation time 24 hours: *Acidovorax avenae subsp. citrulli*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-6.35	-7.94	-9.33	-4.46	-7.72	-7.50	-1.23	-4.81	-2.86	N.D.
70	-9.90	-7.46	0.39	11.5	-5.78	-1.54	-3.93	-0.37	0.20	N.D.
40	-2.96	4.78	-3.08	1.29	-4.20	-6.26	0.43	-39.7	-4.95	N.D.

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 8. Percent growth[1] at incubation time 36 hours: *Acidovorax avenae subsp. citrulli*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-1.75	-4.26	-3.90	-2.53	3.61	8.75	19.7	13.1	13.9	21.6
70	-6.82	-3.76	-5.42	-1.76	-3.28	1.76	-1.56	4.60	0.91	5.08
40	8.79	8.17	14.4	11.3	5.76	1.09	6.74	5.07	6.23	6.35

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 9. Percent growth[1] at incubation time 48 hours: *Acidovorax avenae subsp. citrulli*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-8.92	-10.3	-2.72	-5.66	-5.29	-6.53	-0.82	-2.10	-6.21	-6.73
70	-4.89	-8.08	-2.63	-3.48	-5.70	-8.37	-1.74	-2.44	-3.86	-7.46
40	-0.40	-5.22	-0.18	-2.41	-2.06	-6.50	0.46	-0.40	-0.40	-5.12

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 10. Percent growth[1] at incubation time 60 hours: *Acidovorax avenae subsp. citrulli*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-7.39	-6.82	-5.25	-4.66	-3.25	-1.41	3.59	-0.03	10.21	5.92
70	-9.06	-3.92	-11.0	-5.37	-9.31	-2.48	-6.54	2.61	-5.14	0.56
40	9.76	8.21	5.24	5.18	3.86	6.17	-2.27	4.74	6.19	3.76

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 11. Percent growth[1] at incubation time 12 hours: *Xanthomonas campestris* pv. *campestris*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-3.60	-9.75	-2.55	-4.79	-0.60	0.89	1.46	3.69	12.6	10.7
70	-11.9	-11.5	-9.91	-12.4	-13.2	-8.58	-3.93	0.88	-3.83	-0.51
40	-0.43	-6.26	10.71	1.60	0.26	-5.65	3.43	2.19	3.16	-1.67

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B. 12. Percent growth[1] at incubation time 24 hours: *Xanthomonas campestris* pv. *campestris*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-9.31	-13.9	-5.90	-6.68	-0.64	0.04	8.54	7.04	16.4	17.0
70	-9.54	-7.59	-7.48	-7.40	-6.00	-3.42	-1.65	4.12	0.89	6.07
40	1.33	-0.26	6.79	2.27	-0.18	-2.76	-1.21	-3.49	0.94	-2.08

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B 13. Percent growth[1] at incubation time 36 hours: *Xanthomonas campestris* pv. *campestris*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-4.73	-6.40	-1.78	-5.61	5.57	6.18	21.9	13.0	14.1	20.4
70	-4.97	-2.88	-4.23	-2.42	-1.89	1.66	1.01	5.60	0.68	4.84
40	8.16	5.24	10.5	7.27	3.20	0.54	4.11	1.78	3.59	0.28

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B 14. Percent growth[1] at incubation time 48 hours: *Xanthomonas campestris* pv. *campestris*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-1.75	-4.26	-3.90	-2.53	3.61	8.75	19.7	13.1	13.9	21.6
70	-6.82	-3.76	-5.42	-1.76	-3.28	1.76	-1.56	4.60	0.91	5.08
40	8.79	8.17	14.4	11.3	5.76	1.09	6.74	5.07	6.23	6.35

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.

Table B 15. Percent growth[1] at incubation time 60 hours: *Xanthomonas campestris* pv. *campestris*

FPH TREATMENTS										
	FPH-P			FPH-PA			FPH-T			FPH-C
	1	5.5	10	1	5.5	10	1	5.5	10	
TREATMENT CONCENTRATION (UG/ML)										
100	-7.39	-6.82	-5.25	-4.66	-3.25	-1.41	3.59	-0.03	10.21	5.92
70	-9.06	-3.92	-11.0	-5.37	-9.31	-2.48	-6.54	2.61	-5.14	0.56
40	9.76	8.21	5.24	5.18	3.86	6.17	-2.27	4.74	6.19	3.76

[1] As compared to untreated bacterial/fungal control. Negative values indicate a decrease in growth as compared to control.